CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Substance Name: Mandestrobin

EC Number: ***Not allocated***

CAS Number: ***173662-97-0***

Index Number: **-**

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CONTENTS

Part A.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***Mandestrobin***
EC number:	***Not allocated***
CAS number:	***173662-97-0***
Annex VI Index number:	**-**
Degree of purity:	***Minimum purity 94.0 % w/w (dry weight basis) [based on a pilot plant]***
Impurities:	***No relevant impurities***

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	-
Current proposal for consideration by RAC	Aquatic Acute 1, H400 Aquatic Chronic 1, H410
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Aquatic Acute 1, H400 Aquatic Chronic 1, H410

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	-		-	Conclusive, but not sufficient for classification
2.2.	Flammable gases	-		-	Conclusive, but not sufficient for classification
2.3.	Flammable aerosols	-		-	Conclusive, but not sufficient for classification
2.4.	Oxidising gases	-		-	Conclusive, but not sufficient for classification
2.5.	Gases under pressure	-		-	Conclusive, but not sufficient for classification
2.6.	Flammable liquids	-		-	Conclusive, but not sufficient for classification
2.7.	Flammable solids	-		-	Conclusive, but not sufficient for classification
2.8.	Self-reactive substances and mixtures	-		-	Conclusive, but not sufficient for classification
2.9.	Pyrophoric liquids	-		-	Conclusive, but not sufficient for classification
2.10.	Pyrophoric solids	-		-	Conclusive, but not sufficient for classification
2.11.	Self-heating substances and mixtures	-		-	Conclusive, but not sufficient for classification
2.12.	Substances and mixtures which in contact with water emit flammable gases	-		-	Conclusive, but not sufficient for classification
2.13.	Oxidising liquids	-		-	Conclusive, but not sufficient for classification
2.14.	Oxidising solids	-		-	Conclusive, but not sufficient for classification
2.15.	Organic peroxides	-		-	Conclusive, but not sufficient for classification
2.16.	Substance and mixtures corrosive to metals	-		-	Data lacking
3.1.	Acute toxicity - oral	-		-	Conclusive, but not sufficient for classification
	Acute toxicity - dermal	-		-	Conclusive, but not sufficient for classification
	Acute toxicity - inhalation	-		-	Conclusive, but not sufficient for classification
3.2.	Skin corrosion / irritation	-		-	Conclusive, but not sufficient for classification

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
3.3.	Serious eye damage / eye irritation	-		-	Conclusive, but not sufficient for classification
3.4.	Respiratory sensitisation	-		-	Conclusive, but not sufficient for classification
3.4.	Skin sensitisation	-		-	Conclusive, but not sufficient for classification
3.5.	Germ cell mutagenicity	-		-	Conclusive, but not sufficient for classification
3.6.	Carcinogenicity	-		-	Conclusive, but not sufficient for classification
3.7.	Reproductive toxicity	-		-	Conclusive, but not sufficient for classification
3.8.	Specific target organ toxicity –single exposure	-		-	Conclusive, but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	-		-	Conclusive, but not sufficient for classification
3.10.	Aspiration hazard	-		-	Conclusive, but not sufficient for classification
4.1.	Hazardous to the aquatic environment	H400 H410	M = 1 M = 10	-	-
5.1.	Hazardous to the ozone layer	-		-	Data lacking

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Signal word: Warning!
Pictogram: GHS09
Hazard statements: H410
Precautionary statements: P273, P391, P501

Proposed notes assigned to an entry:

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Mandestrobin has not yet been approved for Annex I under Regulation 1107/2009 (new active substance), with Austria as Rapporteur Member State.

The mammalian toxicology of mandestrobin was discussed in an European expert meeting at EFSA premises (PRAS 122, Nov 2014). The conclusion on the pesticide peer review for mandestrobin was published in the EFSA journal in May 2015 (EFSA Journal 2015;13(5):4100) and contains no proposal for classification and labelling with regard to human health hazards.

In accordance with Article 36(2) of the CLP Regulation, Mandestrobin should be considered for harmonised classification and labelling (including the criteria of the 4th ATP). Therefore, this proposal considers all physico-chemical, human health and environmental end points. This Annex VI dossier presents a classification and labelling proposal based mainly on the information presented in the assessment of Mandestrobin under Regulation 1107/2009. The assessment made under that Regulation is attached to the IUCLID 5 dossier.

Mandestrobin has no harmonised classification and is not listed on Annex VI of the CLP Regulation This proposal seeks for classification and labelling for aquatic toxicity, as proposed during the EFSA peer-review report. No classification is proposed in this CLH report for human health and physico-chemical properties.

2.2 Short summary of the scientific justification for the CLH proposal

For Mandestrobin, no classification and labelling has been proposed regarding physical and chemical properties and human health.

Justification for the proposal with respect to environmental effects:

The active substance S-2200 is a racemic mixture of the *R*-isomer (S-2167) and the *S*-isomer (S-2354). In order to address the fate and behavior of S-2200 in aquatic systems, the hydrolysis, photolysis and biological degradation of both the *R*-isomer and *S*-isomer have been assessed separately. The isomers showed comparable behavior concerning hydrolytic and photolytic degradation, biological degradation and degradation in water sediment systems. No isomerisation between the S-2200 *R*- and *S*- isomers was observed in any of the aquatic studies.

Two hydrolysis studies in dark sterile buffer solutions at pH 4, 7 and 9 at 50 °C using [benzyl-14C] labelled S-2167 and [benzyl-14C] labelled S-2354 were carried out. Both the S-2200 R-isomer and S-2200 S-isomer were hydrolytically stable at pH 4, 7 and 9 at 50°C. According to OECD 111 the expected DT50 at 25°C would be >1 year for each isomer and hence for the racemate, S-2200. No hydrolysis of S-2167 and of S-2354 would be expected under environmental conditions.

Mandestrobin is not readily biodegradable and it cannot be classified as rapidly degraded in water sediment systems since less than 70 % is degraded within 28 days ($DT_{50whole\ system}$ of 212 - 519 days). Furthermore, mineralisation of the active substance is below 10 % of AR after 100 days after application.

Mandestrobin has a low potential of bioaccumulation in aquatic system because of a measured fish BCF of 25-26 (Lentz, N.R., 2010).

Mandestrobin is acute toxic to fish (*Oncorhynchus mykiss*, LC_{50} = 0.94 mg/L, Fournier, A.E., 2009a) and aquatic invertebrates (*Americamysis bahia*, LC_{50} = 0.43 mg/L, Thomas et al., 2012). Mandestrobin is chronic toxic to aquatic invertebrates (*Americamysis bahia*, NOEC = 0.0056 mg/L, Claude et al., 2012).

Combing all these criteria for classification with respect to environmental effects, according to Regulation 1272/2008, *H400, Very toxic to aquatic life* and *H410, Very* t*oxic to aquatic life with long lasting effects,* is proposed for Mandestrobin.

2.3 Current harmonised classification and labelling

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

No current entry in Annex VI, Table 3.1 in the CLP Regulation.

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

No current entry in Annex VI, Table 3.2 in the CLP Regulation.

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

Current self-classification by the applicant Sumitomo Chemical Co. Ltd based on the CLP Regulation criteria: Aquatic Acute Cat. 1 (H400) and Aquatic Chronic Cat. 2 (H411)

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

No need for justification for pesticides.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

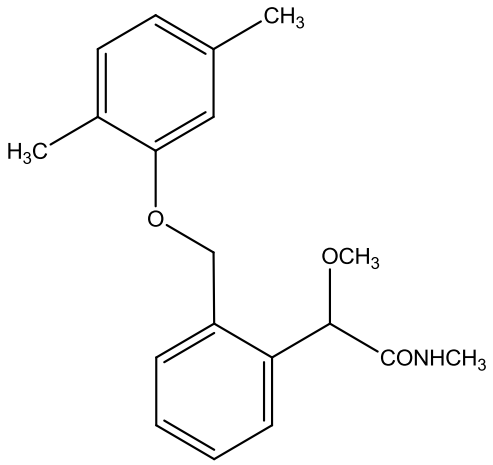
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 4: Substance identity

EC number:	-
EC name:	-
CAS number (EC inventory):	-
CAS number:	173662-97-0
CAS name:	Benzeneacetamide, 2-[(2,5-dimethylphenoxy)methyl]-α-methoxy-*N*-methyl-
IUPAC name:	(*RS*)-2-Methoxy-*N*-methyl-2-[α-(2,5-xylyloxy)-*o*-tolyl]acetamide
CLP Annex VI Index number:	-
Molecular formula:	$C_{19}H_{23}NO_3$
Molecular weight range:	313.39 g/mol

Structural formula:



1.2 Composition of the substance

Table 5: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Mandestrobin	934 g/kg (dry weight basis)	Minimum purity, no range	The minimum purity based on a pilot plant and should be considered provisionally. If commercial production is launched a different minimum purity might be specified.

Current Annex VI entry: no entry

Table 6: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
Xylenes (ortho, meta, para), ethyl benzene	max. 5 g/kg	-	The maximum is based on the technical concentrate (TK)

Current Annex VI entry: -

Table 7: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
No additives	-	-	-	-

Current Annex VI entry: -

1.2.1 Composition of test material

Physico-chemical properties: see table 8 (purity of tested technical material in the range from 90.9% to 100.0%)

Human health hazard assessment: purity of tested technical material was 93.4% (all toxicological and ecotoxicological studies performed with the same batch).

Environmental hazard assessment: purity of tested technical material was 93.4% (all toxicological and ecotoxicological studies performed with the same batch).

1.3 Physico-chemical properties

Table 8: Summary of physico - chemical properties:

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
B.2.1.1 Melting point, freezing point or solidification point (IIA 2.1.1)	EC Method A1 OECD 102 OPPTS 830.7200 (Capillary) GLP	S-2200 PAI Lot: 081103G Purity: 100%	102°C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	107°C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	106°C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.2 Boiling point (IIA 2.1.2)	EC Method A2 OPPTS 830.7220 GLP	S-2200 PAI Lot: 081103G Purity: 100%	296°C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	298°C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	292°C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.3 Temperature of decomposition or sublimation (IIA 2.1.3)			No decomposition was observed. Individual melting and boiling points determined, therefore no sublimation occurred.	Acceptable	
B.2.1.4 Relative density (IIA 2.2)	EC Method A3 OECD 109 OPPTS 830.7300 (Pycnometer) GLP	S-2200 PAI Lot: 081103G Purity: 100%	1.24 g/cm³ at 20.6 °C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	1.23 g/cm³ at 20.6 °C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	1.22 g/cm³ at 20.6 °C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
		S-2200 TGAI Lot: ST-0811G Purity: 93.4%	1.23 g/cm³ at 20.6 °C (mean of three determinations)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2011

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
		S-2200 TK Lot: M1112-2 Purity: 90.9%	1.2015 g/cm³ at 20 °C (mean of five determinations)	Acceptable	Foster, B., 2012a
B.2.1.5 Vapour pressure (IIA 2.3.1)	OECD 104 OPPTS 830.7950 (Gas saturation) GLP	[Benzyl-^{14}C]S-2200 Lot: CFQ40467 Radiochemical purity: 98.9%	Vapour pressure at 20°C: 3.36 x 10^{-8} Pa (extrapolated) Vapour pressure at 25°C: 9.15 x 10^{-8} Pa (extrapolated)	Acceptable	Proctor, K.L. & Lentz, N.R., 2011a
		[Benzyl-^{14}C]S-2200 *R*-isomer Lot: RIS2008-010 Radiochemical purity: 98.78%	Vapour pressure at 20°C: 1.53 x 10^{-6} Pa (extrapolated) Vapour pressure at 25°C: 2.33 x 10^{-6} Pa (extrapolated)	Acceptable	Proctor, K.L. & Lentz, N.R., 2011b
B.2.1.6 Volatility, Henry's law constant (IIA 2.3.2)	calculation		Henry's law constant at 20°C: 6.66 x 10^{-7} Pa.m^3/mol	Acceptable	Liney, P & Jarvis, T., 2012a
B.2.1.7 Appearance: physical state and colour (IIA 2.4.1)	OPPTS 830.6302 OPPTS 830.6303 GLP	S-2200 PAI Lot: 081103G Purity: 100%	Colour: White (Hue: N 9.5/) Physical state: Powdery solid	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	Colour: White (Hue: N 9.5/) Physical state: Powdery solid	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	Colour: White (Hue: N 9.5/) Physical state: Powdery solid	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
		S-2200 TGAI Lot: ST-0811G Purity: 93.4%	Colour: Very pale yellow (Hue: 5Y 9/2) Physical state: Powdery solid	Acceptable	Van Meter, D.S. & Lentz, N.R., 2011
		S-2200 TK Lot: M1112-2 Purity: 90.9%	Colour: White (Hue: N 9.5/) Physical state: Crystalline powdery solid	Acceptable	Foster, B., 2012a
B.2.1.8 Appearance: odour (IIA 2.4.2)	OPPTS 830.6304 GLP	S-2200 PAI Lot: 081103G Purity: 100%	No odour.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	No odour.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	Mild sulfury / acidic odour.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
		S-2200 TGAI Lot: ST-0811G Purity: 93.4%	Mild alcoholic odour.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2011
		S-2200 TK Lot: M1112-2 Purity: 90.9%	No odour.	Acceptable	Foster, B., 2012a
B.2.1.9.1 Spectra of the active substance [UV/VIS] (IIA 2.5.1.1)	UV/VIS OECD 101 OPPTS 830.7050 GLP	S-2200 PAI Lot: 081103G Purity: 100%	Spectra provided in methanol, acidified methanol (10% 1M HCl), basified methanol (10% 1M NaOH) and basified methanol (10% 1M NaOH) neutralised to pH 7 at 25°C. All spectra showed an absorption maximum at 273 nm. Solution / λmax nm / Molar absorptivity, (ε) $(mol/L)^{-1}cm^{-1}$ Methanol 273 2140 Acidic 273 1920 Basic 273 1880 Neutral 273 1740	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	Spectra provided in methanol, acidified methanol (10% 1M HCl), basified methanol (10% 1M NaOH) and basified methanol (10% 1M NaOH) neutralised to pH 7 at 25°C. All spectra showed an absorption maximum at 273 nm. Solution / λmax nm / Molar absorptivity, (ε) $(mol/L)^{-1}cm^{-1}$ Methanol 273 1950 Acidic 273 1980 Basic 273 1530 Neutral 273 1750	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	Spectra provided in methanol, acidified methanol (10% 1M HCl), basified methanol (10% 1M NaOH) and basified methanol (10% 1M NaOH) neutralised to pH 7 at 25°C. All spectra showed an absorption maximum at 273 nm. Solution / λmax nm / Molar absorptivity, (ε) $(mol/L)^{-1}cm^{-1}$ Methanol 273 1990 Acidic 273 1920 Basic 273 1510 Neutral 273 1720	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.9.2 Spectra of the active substance [IR] (IIA 2.5.1.2)	IR EU 91/414/EEC Directive, Annex II, 2.5 GLP	S-2200 PAI Lot: 081103G Purity: 100%	IR spectrum provided and consistent with the structure of S-2200. Wave No. (cm^{-1}) / Assignment 3394 N-H stretch 3057 Aromatic C-H stretch 2919, 2833 Aliphatic C-H stretch 1670 C=O stretch	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	IR spectrum provided and consistent with the structure of S-2200 *R*-isomer. Wave No. (cm^{-1}) / Assignment 3405 N-H stretch 3059 Aromatic C-H stretch 2939, 2831 Aliphatic C-H stretch 1670 C=O stretch	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	IR spectrum provided and consistent with the structure of S-2200 *S*-isomer. Wave No. (cm^{-1}) / Assignment 3405 N-H stretch 3060 Aromatic C-H stretch 2939, 2831 Aliphatic C-H stretch 1670 C=O stretch	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.9.3 Spectra of the active substance [NMR]	NMR EU 91/414/EEC Directive, Annex II, 2.5	S-2200 PAI Lot: 081103G Purity: 100%	^{1}H and ^{13}C NMR spectra provided and consistent with the structure of S-2200. This is demonstrated by the chemical shift peak assignments.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
(IIA 2.5.1.3)	GLP	S-2200 *R*-isomer Lot: 060020652 Purity: 100%	^{1}H and ^{13}C NMR spectra provided and consistent with the structure of S-2200 *R*-isomer. This is demonstrated by the chemical shift peak assignments.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	^{1}H and ^{13}C NMR spectra provided and consistent with the structure of S-2200 *S*-isomer. This is demonstrated by the chemical shift peak assignments.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.9.4 Spectra of the active substance [MS] (IIA 2.5.1.4)	MS EU 91/414/EEC Directive, Annex II, 2.5 GLP	S-2200 PAI Lot: 081103G Purity: 100%	Spectra produced using high performance liquid chromatography with mass spectrometry (HPLC/MS) were consistent with the structure of S-2200. The fragmentation pattern for the S-2200 PAI was identical to the fragmentation patterns for the two individual isomers (S-2200 *R*-isomer and S-2200 *S*-isomer), confirming that the S-2200 PAI is a racemic mixture of the two isomers. Two primary fragments and two secondary fragments were observed and identities were proposed. Three adducts, corresponding to the hydrogen, sodium and potassium adducts of the molecular ion, were observed in each of the mass spectra. Peaks of *m*/*z* 314, 336, 352 were observed, which were consistent with hydrogen, sodium and potassium adducts of the molecular ion, respectively.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	Spectra produced using high performance liquid chromatography with mass spectrometry (HPLC/MS) were consistent with the structure of S-2200 *R*-isomer. Two primary fragments and two secondary fragments were observed and identities were proposed. Three adducts, corresponding to the hydrogen, sodium and potassium adducts of the molecular ion, were observed in the mass spectrum. Peaks of *m*/*z* 314, 336, 352 were observed, which were consistent with hydrogen, sodium and potassium adducts of the molecular ion, respectively.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	Spectra produced using high performance liquid chromatography with mass spectrometry (HPLC/MS) were consistent with the structure of S-2200 *S*-isomer. Two primary fragments and two secondary fragments were observed and identities were proposed. Three adducts, corresponding to the hydrogen, sodium and potassium adducts of the molecular ion, were observed in the mass spectrum. Peaks of *m*/*z* 314, 336, 352 were observed, which were consistent with hydrogen, sodium and potassium adducts of the molecular ion, respectively.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.9.5 Wavelengths at which UV/VIS molecular extinction occurs, where appropriate, to include a wavelength at the highest absorption above 290 nm (IIA 2.5.1.5)	UV/VIS OECD 101 GLP	S-2200 PAI Lot: 081103G Purity: 100%	Measurements up to 800 nm show no more absorptions as reported in B.2.1.9.1.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%			Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%			Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.9.6 Optical purity (IIA 2.5.1.6)			The PAI, TGAI and TK consist of a racemic mixture of the R and the S isomer.	Acceptable	
B.2.1.10 Spectra of relevant impurities (IIA 2.5.2)			Not relevant since no impurities of toxicological or environmental concern are stated.	Acceptable	
B.2.1.11 Solubility in water (IIA 2.6)	OPPTS 830.7840 OECD 105 EC Method A6 JMAFF 8147 (shake flask) GLP	[Benzyl-^{14}C]S-2200 Lot: CFQ15155 Radiochemical purity: 100%	Water solubility at 20°C = 15.8 mg/L The effect of pH on water solubility was not determined as S-2200 does not dissociate under acidic or basic conditions.	Acceptable	Lentz, N.R., & Van Meter, D.S., 2009a
		[Benzyl-^{14}C]S-2200 *R*-isomer Lot: RIS2008-010 Radiochemical purity: 99.2%	Water solubility at 20°C = 25.8 mg/L The effect of pH on water solubility was not determined as S-2200 *R*-isomer does not dissociate under acidic or basic conditions.	Acceptable	Lentz, N.R., & Van Meter, D.S., 2009b

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
		[Benzyl-^{14}C]S-2200 *S*-isomer Lot: RIS2008-009 Radiochemical purity: 98.34%	Water solubility at 20°C = 29.1 mg/L The effect of pH on water solubility was not determined as S-2200 *S*-isomer does not dissociate under acidic or basic conditions.	Acceptable	Lentz, N.R., & Van Meter, D.S., 2009c
B.2.1.12 Solubility in organic solvents (IIA 2.7)	OPPTS 830.7840 OECD 105 EC Method A6 (shake flask) GLP	S-2200 PAI Lot: 081103G Purity: 100%	Solubilities at 20°C: Acetone 275 g/L Dichloromethane 522 g/L Ethyl acetate 158 g/L Hexane 1.46 g/L Methanol 169 g/L n-Octanol 31.8 g/L Toluene 114 g/L	Acceptable	Lentz, N.R., & Van Meter, D.S., 2011a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	Solubilities at 20°C: Acetone 424 g/L Dichloromethane 519 g/L Ethyl acetate 264 g/L Hexane 2.57 g/L Methanol 352 g/L n-Octanol 97.7 g/L Toluene 227 g/L	Acceptable	Lentz, N.R., & Van Meter, D.S., 2011b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	Solubilities at 20°C: Acetone 431 g/L Dichloromethane 577 g/L Ethyl acetate 266 g/L Hexane 2.79 g/L Methanol 387 g/L n-Octanol 83.9 g/L Toluene 216 g/L	Acceptable	Lentz, N.R., & Van Meter, D.S., 2011c
		S-2200 TGAI Lot: ST-0811G Purity: 93.4%	Solubilities at 20°C: Acetone 310 g/L Dichloromethane 480 g/L Ethyl acetate 186 g/L Hexane 2.49 g/L Methanol 217 g/L n-Octanol 30.8 g/L Toluene 128 g/L	Acceptable	Lentz, N.R., & Van Meter, D.S., 2010a
B.2.1.13.1 Partition coefficient	OPPTS 830.7550 OECD 107 EC Method A8	S-2200 PAI Lot: 081103G Purity: 100%	Log P_{ow} at 25 ± 1°C = 3.51 (P_{ow} = 3240)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010d

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
n-octanol/water (IIA 2.8.1)	JMAFF 8147 (shake flask) GLP	S-2200 *R*-isomer Lot: 060020652 Purity: 100%	Log P_{ow} at 25 ± 1°C = 3.44 (P_{ow} = 2730)	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010e
B.2.1.13.2 Effect of pH (4-10) on the n-octanol/water partition co-efficient (IIA 2.8.2)			The effect of pH on partition coefficient was not determined as S-2200 does not dissociate under acidic or basic conditions.	Acceptable	
B.2.1.14 Hydrolysis rate (IIA 2.9.1)	OPPTS 835.2110 EC Method C7 OECD 111 JMAFF 8147, section 2-6-1 and 2-9-13 GLP	[Benzyl-^{14}C]S-2200 *R*-isomer Lot: RIS2008-010 Radiochemical purity: 99.2%	S-2200 *R*-isomer was stable to hydrolysis at pH 4, 7 and 9. No degradation products were detected and no conversion of S-2200 *R*-isomer to S-2200 *S*-isomer was observed. DT50 at pH 4, 7 and 9 at 25ºC: > 1 year, as < 10% of the S-2200 *R*-isomer degrades for 5 days at 50ºC. No hydrolysis of S-2200 *R*-isomer would therefore be expected under normal environmental conditions.	Acceptable	Lewis, C.J. & Alderman, D., 2010a
		[Benzyl-^{14}C]S-2200 *S*-isomer Lot: RIS2008-009 Radiochemical purity: 99.4%	S-2200 *S*-isomer was stable to hydrolysis at pH 4, 7 and 9. No degradation products were detected and no conversion of S-2200 *S*-isomer to S-2200 *R*-isomer was observed. DT50 at pH 4, 7 and 9 at 25ºC: > 1 year, as < 10% of the S-2200 *S*-isomer degrades for 5 days at 50ºC. No hydrolysis of S-2200 *S*-isomer would therefore be expected under normal environmental conditions.	Acceptable	Lewis, C.J. & Alderman, D., 2010b

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
B.2.1.15 Direct phototrans-formation (IIA 2.9.2)	OECD 316 EPA Pesticide Assessment Guidelines, Subdivision N, Section 161-2 JMAFF 8147, section 2-6-2 GLP	[Benzyl-^{14}C] S-2200 *R*-isomer Lot: RIS2008-010 Radiochemical purity: 99.2% [Phenoxy-^{14}C] *S*-2200 *R*-isomer Lot: RIS2009-002 Radiochemical purity: 99.1%	Photodegradation of S-2200 R-isomer in sterile pH 7 buffer at 25 ºC : Label DT_{50} (days) DT_{75} (days) DT_{90} (days) Benzyl 5.3 10.5 17.5 Phenoxy 3.6 7.2 12.0 S-2200-OR and S-2200-ORC were identified as the major degradation products at maximum levels of 23% and 14% of applied radioactivity, respectively. Two other degradation products, S-2200-PR and De Xy-2200, were present in the approximate range of 5 to 10%. A large number of minor unknowns were present at <10% of applied radioactivity. The DT50's for two of the degradation products were calculated to be: Analyte DT_{50} (days) S-2200-OR 5.1 S-2200-PR 2.5	Acceptable	Lewis, C.J. & Alderman, D., 2010c
		[Benzyl-^{14}C]S-2200 *S*-isomer Lot: RIS2008-009 Radiochemical purity: >98%	Photodegradation of S-2200 S-isomer in sterile pH 7 buffer at 25 ºC : Label DT_{50} (days) DT_{75} (days) DT_{90} (days) Benzyl 4.6 9.2 15.3 S-2200-OR and S-2200-ORC were identified as the major degradation product at maximum levels of 18.6% and 10.5% of applied radioactivity, respectively. Two other degradation products, S 2200-PR and De Xy-2200, were present in the approximate range of 5 to 10%. A large number of minor unknowns were present at <10% of applied radioactivity. The DT50's for two of the degradation products were calculated to be: Analyte DT50 (days) S-2200-OR 4.0 S-2200-PR 2.2	Acceptable	Lewis, C.J. & Alderman, D., 2010d

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
B.2.1.16 Quantum yield (IIA 2.9.3)	OECD 316 EPA Pesticide Assessment Guidelines, Subdivision N, Section 161-2 JMAFF 8147, section 2-6-2 GLP	[Phenoxy-^{14}C] *S*-2200 *R*-isomer Lot: RIS2009-002 Radiochemical purity: 99.1%	Quantum yield in pH 7 buffer: 0.283	Acceptable	Lewis, C.J. & Alderman, D., 2010c
		[Benzyl-^{14}C]S-2200 *S*-isomer Lot: RIS2008-009 Radiochemical purity: >98%	Quantum yield in pH 7 buffer: 0.269	Acceptable	Lewis, C.J. & Alderman, D., 2010d
B.2.1.17 Lifetime in the top layer of aqueous systems (IIA 2.9.4)	Calculation only		Calculated photolytic half-life values of S-2200 using GCSOLAR programme are 1.20 – 3.03 days at latitudes 30, 40 and 50°N in summer.	Acceptable	Nishimura, H., Fujisawa, T., Katagi, T., 2012
B.2.1.18 Dissociation constant (pKa) (IIA 2.9.5)	UV/VIS OECD 101 OPPTS 830.7050 GLP	S-2200 PAI Lot: 081103G Purity: 100%	No spectral shift was observed in the S-2200 peak maxima at 273 nm, in any of the solutions tested. The solutions ranged from pH 2 to pH 10. This demonstrates that S-2200 has no dissociative activity in this pH range.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010a
		S-2200 *R*-isomer Lot: 060020652 Purity: 100%	No spectral shift was observed in the S-2200 *R*-isomer peak maxima at 273 nm, in any of the solutions tested. The solutions ranged from pH 2 to pH 10. This demonstrates that S-2200 *R*-isomer has no dissociative activity in this pH range.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010b
		S-2200 *S*-isomer Lot: 060020653 Purity: 99.7%	No spectral shift was observed in the S-2200 *S*-isomer peak maxima at 273 nm, in any of the solutions tested. The solutions ranged from pH 2 to pH 10. This demonstrates that S-2200 *S*-isomer has no dissociative activity in this pH range.	Acceptable	Van Meter, D.S. & Lentz, N.R., 2010c
B.2.1.19 Stability in air, photochemical oxidative degradation (IIA 2.10)	AOPWIN (v1.92) Prediction	--	The decomposition rate constant of S-2200 in air following reaction with hydroxyl radicals was calculated to be 96.3802 x 10^{-12} cm^3 $molecule^{-1}sec^{-1}$. Assuming that the 12 hour daytime hydroxyl radical concentration is 1.5 x 10^6 $molecules.cm^{-3}$, the half-life was calculated to be 0.111 days (12 hr day) or 1.332 hrs. No ozone reaction could be estimated.	Acceptable	Liney, P & Jarvis, T., 2012b

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
B.2.1.20 Flammability (IIA 2.11.1)	EEC Method A.10 GLP	S-2200 TGAI Lot: ST-0811G Purity: 93.4%	In the preliminary screen S-2200 did not ignite. Not classified as flammable.	Acceptable according to Directive 67/548/EEC. The result is acceptable according to Regulation 1272/2008/EEC as well. No classification.	Foster, B. & Heslop, D., 2012
B.2.1.21 Auto-flammability (IIA 2.11.2)	EEC Method A.16 - Relative self-ignition temperature GLP	S-2200 TGAI Lot: ST-0811G Purity: 93.4%	No auto-ignition was observed up to 400°C.	Acceptable according to Directive 67/548/EEC. According to the Regulation 1272/2008/EEC no test procedure is required if the substance is completely molten at 160 °C. This is demonstrated in B.2.1.1. No classification.	Lentz, N.R., 2010
B.2.1.22 Flash point (IIA 2.12)			Not required, as the Mandestrobin substance is a solid with a melting point above 40°C.		
B.2.1.23 Explosive properties (IIA 2.13)	OPPTS 830.6316 OECD 113 (thermal explodability) GLP	S-2200 TGAI Lot: ST-0811G Purity: 93.4%	Explosive screen by DSC: An endotherm was observed, which corresponded to the melting point of S-2200 and was not due to thermal instability. No exotherm was observed, therefore S-2200 is considered to not possess explosive properties between 20 and 500°C.	Additional information Test is not according to EEC A14.	Lentz, N.R., & Van Meter, D.S., 2011d
	EC Method A14 OPPTS 830.6316 (impact explodability) GLP	S-2200 TGAI Lot: ST-0811G Purity: 93.4%	S-2200 displayed no explosive properties during the mechanical sensitivity tests. S-2200 can be considered to possess no explosive properties.	Acceptable Mandestrobin is considered having no explosive properties according to Directive 67/548/EEC. The statement is acceptable according to Regulation 1272/2008/EEC as well. No classification.	Lentz, N.R., & Van Meter, D.S., 2010b
B.2.1.24 Surface tension (IIA 2.14)	EC Method A5 OECD 115 GLP	S-2200 TGAI Lot: ST-0811G Purity: 93.4%	Surface tension: 61.2 mN/m at a concentration of 90% of the saturation solubility and 20 ºC.	Acceptable	Foster, B., 2012b

Study	Method	Material / Batch	Results	Conclusion/Comment	Reference
B.2.1.25 Oxidizing properties (IIA 2.15)	EC Method A17 GLP	S-2200 TGAI Lot: ST-0811G Purity: 93.4%	The maximum burning rate of S-2200 was lower than that of the reference substance. Therefore, S-2200 does not possess oxidizing properties.	Acceptable according to Directive 67/548/EEC. The result is acceptable according to Regulation 1272/2008/EEC as well. No classification.	Foster, B., 2011
B.2.1.2.26 pH (IIA 2.16)			Not required for EU		
B.2.1.2.27 Storage stability (IIA 2.17.1)			Not required for EU		
B.2.1.2.28 Stability (temperature, metals) (IIA 2.17.2)			Not required for EU		
B.2.1.2.29 Other/special studies (IIA 2.18)			None		

2 MANUFACTURE AND USES

2.1 Manufacture

Not relevant for Classification and Labelling.

2.2 Identified uses

S-2200 is a strobilurin fungicide to be used for control of *Sclerotinia*. It can translocate in plants via translaminar movement and systemic transportation. It acts by inhibiting mitochondrial respiration in fungi. Strobilurin fungicides bind at the Qol-centre on cytochrome b and block electron transfer between cytochrome b and cytochrome c1. This disrupts the energy cycle within the fungus by halting the production of ATP.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Mandestrobin has been adequately tested for physico-chemical properties. It is not explosive, oxidising, flammable or auto-flammable and does not fulfil the classification criteria for physico-chemical properties. Therefore, no classification is required.

4 HUMAN HEALTH HAZARD ASSESSMENT

Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

Absorption, distribution, excretion and metabolism (toxicokinetics):

Absorption and Excretion

Following a single oral high (1000 mg/kg bw) or low (5 mg/kg bw) dose administration of racemic [^{14}C]-mandestrobin, labelled at either the benzyl or phenoxy rings, radioactivity was rapidly absorbed (more rapid at low than high doses). At the low dose, absorption of mandestrobin, calculated from the radioactivity recovered in urine and bile, was greater than 90% of the administered dose. At the high dose, systemic exposure (AUC) to mandestrobin derived radioactivity was sub-proportional, indicating saturation of absorption processes following administration. Secondary absorption phases following administration of 1000 mg/kg doses of mandestrobin indicated that systemic exposure at high concentrations may have resulted from one or more of the following: (a) enterohepatic recirculation, (b) differential absorption during transit through the gastro-intestinal tract, or (c) transient solubility of mandestrobin. There were no significant gender differences in the pharmacokinetics of mandestrobin at 5 or 1000 mg/kg bodyweight.
Clearance from the plasma was almost complete by 120 hours post-administration after a single oral dose of 5 or 1000 mg/kg bodyweight. Faecal elimination, via the bile, was the primary route of elimination of radioactivity. However, renal elimination was also important for the excretion of metabolites. More than 70% of radioactivity was eliminated within 48 hours after single oral administration. There did not appear to be any gender-, dose- or radiolabel- related differences in either the rates or routes of excretion.

In an investigation of ADME of the two stereo isomers of the active substance, coded S-2200 *R*-isomer and S-2200 *S*-isomer, both were absorbed immediately after administration. S-2200 *R*-isomer derived radioactivity was rapidly excreted after single oral administration, whereas excretion of radiolabel from S-2200 *S*-isomer was less rapid (likely due to enterohepatic recirculation). In this study, urinary excretion was greater in female than male rats for both isomers. Excretion into expired air was negligible.

Following the repeated daily administration of [benzyl-^{14}C]-mandestrobin at 5 mg/kg bw for 14 days, there were no differences compared to single dose of 5 mg/kg bw. Faeces was again the primary route of excretion.

Distribution

Following oral exposure, mandestrobin is widely distributed throughout the body. After single oral administration there was no major difference in distribution between high and low doses

(1000 and 5 mg/kg bw, respectively), or between sexes. The major tissue residues were seen in the gastrointestinal tract, and in liver and kidney, as well as in uterus and ovaries at 168 hours after dosing. Tissue distribution was similar in animals administered [benzyl-^{14}C]-mandestrobin or [phenoxy-^{14}C]-mandestrobin indicating that the core of the molecule was stable during systemic exposure. Furthermore, a similar distribution of radioactivity into tissues was observed following 1, 6, 10 and 14 daily doses of 5 mg/kg bw. There was no evidence of accumulation into tissues.

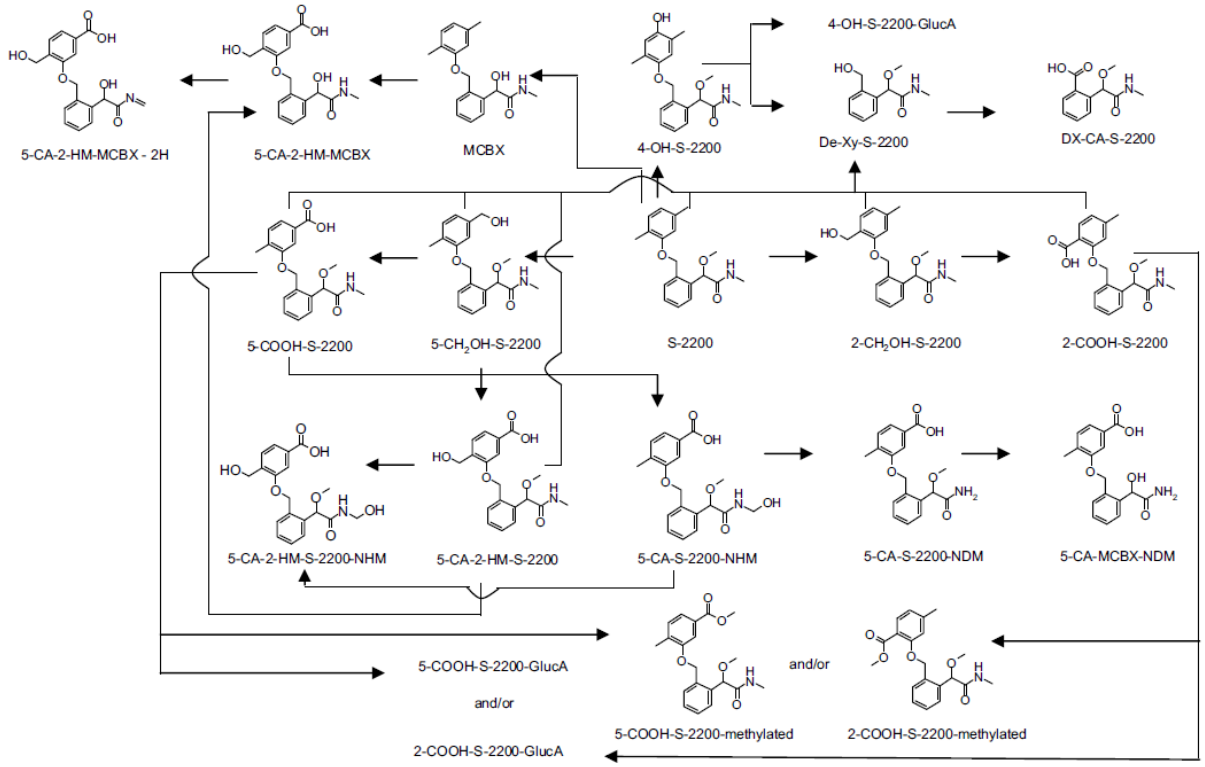
Metabolism

Mandestrobin was extensively metabolised to numerous metabolites. Unchanged parent was found in faeces only at < 0.2% of administered dose after a single low dose of 5 mg/kg bw and at < 6% after single high dose of 1000 mg/kg bw. The primary routes of metabolism were by (i) oxidation and subsequent conjugation with glucuronic acid, (ii) demethylation with subsequent oxidation, or (iii) oxidation with subsequent demethylation. Metabolite fractions in plasma, liver and kidney were identified. Residues were in general readily extractable from tissue matrix. There was no discernible shift in radiolabel distribution between low and high dose.

In an investigation of ADME of the two stereo isomers of the active substance, coded S-2200 *R*-isomer and S-2200 *S*-isomer, twelve metabolites were identified and quantified: 5-CA-S-2200-NHM was the predominant metabolite of the S-2200 *R*-isomer. 4-OH-S-2200, followed by 5-COOH-S-2200, were the major metabolites of S-2200 *S*-isomer. However, the same metabolites were identified both in S-2200 *R*-isomer and *S*-isomer. The “A” glucuronide of 4-OH-S-2200 (4-OH-S-2200-Glucuronide A) is likely to be subject to enterohepatic circulation. This metabolic pathway is likely to occur more commonly with S-2200 *S*-isomer, because the rate of radiolabel excretion was slower (delayed by enterohepatic recirculation) than that for S-2200 *R*-isomer.

Following repeated administration of radiolabelled S-2200 at 5 mg/kg bw for 14 days, metabolism was similar to that after single dose, however, kidney profiles exhibited both gender and repeat dose differences, in the type and number of metabolites observed.

Proposed metabolic pathway of mandestrobin (S-2200) in rats:



Dermal absorption: An *in vitro* dermal absorption study with human skin has been conducted to determine levels of absorption associated with exposure to the concentrate and to the aqueous spray dilution (1/5000 v/v) of the product "S-2200 25SC". According to the 2012 EFSA guidance, the dermal absorption values for mandestrobin contained in the formulation "S-2200 25SC" are 0.1% for the concentrate and 8% for the dilution.

Table 9: Summary table of relevant dermal absorption studies

Method	Results	Remarks	Reference
In vitro dermal absorption (OECD 428)	"S-2200 25 SC" Concentrate: 0.1% Spray dilution (1/5000 v/v): 8%	Human epidermis	Hadfield, N.; 2011

4.1.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.1.3 Summary and discussion on toxicokinetics

Rate and extent of oral absorption ‡	> 95% based on urinary and biliary excretion within 24 hours
Distribution ‡	Extensively distributed throughout the body (mainly in gastro-intestinal tract, liver and kidneys)
Potential for accumulation ‡	No evidence for accumulation
Rate and extent of excretion ‡	Faecal elimination, via the bile, was the primary route of elimination (~80%), urinary excretion up to ~15%)
Metabolism in animals ‡	Extensively metabolised to numerous metabolites (unchanged parent in faeces < 0.2% of low dose). The primary routes of metabolism were by oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, or oxidation with subsequent demethylation.

4.2 Acute toxicity

Table 10: Summary table of relevant acute toxicity studies with mandestrobin

Method	Results	Remarks	Reference
Oral route (OECD 423)	♀ LD_{50} > 2000 mg/kg bw	Wistar rats (Slc:WistarHannover/ Rcc) Purity 93.4%	*Asano H.; 2010a*
Dermal route (OECD 402)	♂/♀ LD_{50} > 2000 mg/kg bw	Wistar rats (Slc:WistarHannover/ Rcc) Purity 93.4%	*Asano H.; 2010b*
Inhalation route (OECD 403)	♂/♀ LC_{50} > 4.964 mg/L (maximum attainable concentration)	Wistar rats (Slc:WistarHannover/ Rcc), 4 hours, nose only Purity 93.4%	*Deguchi Y.; 2010*

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

No mortality was observed. Clinical signs observed during the observation period included stains around the anus and liquid stools containing a white compound. Clinical signs appeared from 4 hours after administration and had disappeared on day 1. The body weights were not affected by the administration of the test compound throughout the study period. There were no findings at the gross pathological examination.

The oral LD_{50} of mandestrobin in female rats was > 2000 mg/kg bw (limit test). According to Regulation (EC) No. 1272/2008, classification of mandestrobin for acute oral toxicity is not required.

4.2.1.2 Acute toxicity: inhalation

No mortality and no treatment related clinical signs were observed during treatment and observation period. Wet fur was observed in both sexes of air control and treatment groups immediately after the exposure period, but disappeared within two hours post-dosing. This finding is considered to be a result of the restraint procedure and not of toxicity related to treatment. Body weights in the treatment groups were comparable to controls. No lesions were detected at the gross pathological examination at the end of the study period.

The 4-hour inhalation LC_{50} was determined to be > 4.96 mg/L (maximum attainable concentration) for male and female rats. According to Regulation (EC) No. 1272/2008, classification of mandestrobin for acute inhalation toxicity is not required.

4.2.1.3 Acute toxicity: dermal

No mortality was observed, and all animals appeared normal throughout the study. Body weight was not affected by administration of the test compound. There were no findings at the gross pathological examination. No dermal irritation was recorded in this study.

The dermal LD_{50} of mandestrobin in male and female rats was > 2000 mg/kg bw. According to Regulation (EC) No. 1272/2008, classification of mandestrobin for acute dermal toxicity is not required.

4.2.1.4 Acute toxicity: other routes

No data on other routes available.

4.2.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.2.3 Summary and discussion of acute toxicity

Mandestrobin is of low acute oral, dermal and inhalation toxicity (oral LD_{50} > 2000 mg/kg bw, dermal LD_{50} > 2000 mg/kg bw, LC_{50} > 4.96 mg/L air) in rats.

4.2.4 Comparison with criteria

All estimated LD_{50} and LC_{50} values of mandestrobin are above the criteria for classification and labelling (CLP Regulation).

4.2.5 Conclusions on classification and labelling

No classification and labelling is proposed for mandestrobin regarding acute toxicity.

4.3 Specific target organ toxicity – single exposure (STOT SE)

The hazard class STOT SE covers three sub-categories. Categories 1 and 2 are assigned for non-lethal “significant and/or severe toxic effects”, reflecting the dose level required to cause the effect. Category 3 covers “transient effects” occurring after single exposure, specifically respiratory tract irritation (RTI) and narcotic effects (NE).

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

No human epidemiology data are available. No health effects have been recorded in workers and no poisoning incidents or clinical cases have been reported. In addition, no specific, non lethal, target organ toxicity after single exposure to mandestrobin was observed in acute toxicity studies

and acute neurotoxicity study. Mandestrobin is of low toxicity after a single exposure, by all routes of administration. There were no indications of respiratory tract irritation (RTI) and narcotic effects (NE) that could conceivably be elicited by a single dose or exposure to mandestrobin.

In the acute inhalation toxicity study in rats, no signs of irritation on respiratory tract were observed. No respiratory tract irritation in members of staff involved in the synthesis and development of mandestrobin (who are routinely monitored) has been detected by, or reported to, medical staff.

4.3.2 Comparison with criteria

There were no effects observed in acute toxicity studies and in an acute neurotoxicity study that would trigger classification and labelling as STOT SE.

Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single exposure should be classified as STOT SE 1 or 2 according to the CLP Regulation. Classification should be supported by evidence associating single exposure to the substance with a consistent and identifiable toxic effect that clearly impacts health. Classification in STOT SE 3 is reserved for transient target organ effects and is limited to substances that have narcotic effects or cause respiratory tract irritation.

Mandestrobin does not induce clinical signs in the available acute oral and dermal toxicity studies, and in the acute inhalation study only a general sign of toxicity was seen. All effects in animals noted were recorded below lethal doses on the day of dosing, and resolved quickly thereafter, without causing specific target organ toxicity. Therefore, there is no need to classify mandestrobin for STOT SE 1 or 2. Furthermore there was no evidence of respiratory irritation following exposure to mandesrobin that would warrant a classification as STOT SE 3. No narcotic effects were noted. No human data support the need to classify mandestrobin for STOT SE.

4.3.3 Conclusions on classification and labelling

No classification and labelling is proposed for mandestrobin regarding specific target organ toxicity after single exposure.

4.4 Irritation

4.4.1 Skin irritation

Table 11: Summary table of relevant skin irritation studies

Method	Results	Remarks	Reference
Primary skin irritation (OECD 404)	Not irritating	New Zealand White Rabbit Purity: 93.4%	Ota, M.; 2010a

4.4.1.1 Non-human information

No signs of ill health or toxicity were observed in any of the animals during the experimental period. No skin irritation reactions were observed in any animal at any timepoint during the observation period.

The dermal irritation score was 0.0 (non-irritating). According to Regulation (EC) No. 1272/2008, classification of mandestrobin as skin irritant is not required.

4.4.1.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.4.1.3 Summary and discussion of skin irritation

According to the results of the rabbit skin irritation study, mandestrobin is not irritant to the intact shaved rabbit skin.

4.4.1.4 Comparison with criteria

Estimated skin irritation scores (0.00) are below the criteria triggering classification and labelling (according to CLP).

4.4.1.5 Conclusions on classification and labelling

No classification and labelling is proposed for mandestrobin regarding skin irritation.

4.4.2 Eye irritation

Table 12: Summary table of relevant eye irritation studies

Method	Results	Remarks	Reference
Eye irritation (OECD 405)	Slightly irritating	New Zealand White rabbit Purity: 93.4%	Ota, M.; 2010b

4.4.2.1 Non-human information

No signs of ill health or toxicity were observed in any of the animals during the experimental period. One hour after application, conjunctival redness (grade 1), chemosis (grade 1), and discharge (grade 1) was observed in all three rabbits, and congestion (grade 1) in the iris was observed in 2 out of 3 animals in the unwashed group. 24 hours after application, opacity (grade 1) of the cornea and conjunctival redness (grade 1) were observed in all 3 rabbits, and 2 out of 3

rabbits showed chemosis (grade 1) and discharge (grade 1-2) in the conjunctiva. 48 hours after application, corneal opacity (grade 1) was observed in one animal. All ocular findings were reversible within 72 hours after application.

In the washed group, the following ocular findings were present at the 24 hour timepoint: redness grade 1 in all three animals, chemosis grade 1 in one animal. There were no findings at later timepoints.

Table 13: Eye irritation scores (unwashed group)

		Rabbit Nr.	1 hour	24 hours	48 hours	72 hours	Mean/Rabbit (24 h + 48 h + 72 h)
Conjunctiva	**Chemosis**	**1**	1	1	0	0	0.33
		2	1	1	0	0	0.33
		3	1	0	0	0	0.00
	Redness	**1**	1	1	0	0	0.33
		2	1	1	0	0	0.33
		3	1	1	0	0	0.33
Iris	**Congestion**	**1**	0	0	0	0	0.00
		2	1	0	0	0	0.00
		3	1	0	0	0	0.00
Cornea	**Opacity**	**1**	0	1	1	0	0.67
		2	0	1	0	0	0.33
		3	0	1	0	0	0.33

Table 14: Eye irritation scores (washed group)

		Rabbit Nr.	1 hour	24 hours	48 hours	72 hours	Mean/Rabbit (24 h + 48 h + 72 h)
Conjunctiva	**Chemosis**	**4**	1	0	0	0	0.00
		5	1	0	0	0	0.00
		6	1	1	0	0	0.33
	Redness	**4**	1	1	0	0	0.33
		5	1	1	0	0	0.33
		6	1	1	0	0	0.33
Iris	**Congestion**	**4**	0	0	0	0	0.00
		5	0	0	0	0	0.00
		6	0	0	0	0	0.00
Cornea	**Opacity**	**4**	0	0	0	0	0.00

		Rabbit Nr.	1 hour	24 hours	48 hours	72 hours	Mean/Rabbit (24 h + 48 h + 72 h)
		5	0	0	0	0	0.00
		6	0	0	0	0	0.00

Thus, mandestrobin caused only mild transient ocular irritation in rabbits.

4.4.2.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.4.2.3 Summary and discussion of eye irritation

According to the results of an eye irritation study in rabbits, mandestrobin is slightly irritating to the eyes; according to classification criteria, classification and labelling is not warranted.

4.4.2.4 Comparison with criteria

The observed eye irritation scores (24 – 72 hours; 0.22 (chemosis), 0.33 (conjunctival redness), 0.00 (iris congestion) and 0.44 (corneal opacity)) are below the criteria for triggering classification and labelling (according to CLP).

4.4.2.5 Conclusions on classification and labelling

No classification and labelling is proposed for mandestrobin regarding eye irritation.

4.5 Corrosivity

Mandestrobin did not show any corrosive properties in rabbit skin and eye irritation studies (see 4.4.1 and 4.4.2).

4.6 Sensitisation

4.6.1 Skin sensitisation

Table 15: Summary table of relevant skin sensitisation studies

Method	Results	Remarks	Reference
Skin sensitization (M&K-test) (OECD 406)	Not sensitising	SIc:Hartley Guinea pig Purity: 93.4%	Ota, M.; 2010c

4.6.1.1 Non-human information

No signs of ill health or toxicity were observed in any of the animals during the experimental period. The body weights of all animals increased normally during the experimental period.

After challenge, no dermal reaction was observed in any of the 20 animals of the test group. No skin reactions were observed in the control groups. Slight to moderate erythema and oedema were observed in all 5 animals treated with the positive control HCA. From these results the sensitisation rate for mandestrobin was estimated to be 0%.

Mandestrobin does not require classification and labelling for sensitization according to 1272/2008/EEC.

4.6.1.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.6.1.3 Summary and discussion of skin sensitisation

According to the results of the skin sensitisation study in guinea pigs (Maximisation test), mandestrobin is not sensitising to the skin; according to classification criteria, classification and labelling is not warranted.

4.6.1.4 Comparison with criteria

Effects observed in the skin sensitisation study on guinea pigs are below the criteria for triggering classification and labelling (according to CLP).

4.6.1.5 Conclusions on classification and labelling

No classification and labelling is proposed for mandestrobin regarding skin sensitisation.

4.6.2 Respiratory sensitisation

No data on respiratory sensitisation are available.

4.7 Repeated dose toxicity

Table 16: Summary table of relevant repeated dose toxicity studies

Method	Dose range / NOAEL /Effects	Remarks	Reference
Rat, 90-days (oral) (OECD 408)	0, 800, 4000, 10000, 20000 ppm equivalent to: ♂: 0, 54, 282.6, 742.7, 1544.6 mg/kg bw/day ♀: 0, 61.6, 320.1, 788.5, 1886.5 mg/kg bw/day <u>NOAEL:</u> ♂ 800 ppm (54 mg/kg bw/day) ♀ 4000 ppm (320.1 mg/kg bw/day) <u>Effects at LOAEL</u>: ♂: ↑ absolute and relative liver weight ♀: ↑ absolute and relative liver weight Hepatocellular hypertrophy Follicular cell hypertrophy in the thyroid ↑ Cholesterol levels	Wistar; Crl:WI(Han) rats Purity: 93.4%	Beck, W.; 2011a
Mouse, 90-days (oral) (OECD 408)	0, 1750, 3500, 7000 ppm equivalent to: ♂: 0, 204.1, 404.9, 807.3 mg/kg bw/day ♀: 0, 251.8, 529.1, 1111.2 mg/kg bw/day <u>NOAEL:</u> 7000 ppm ♂ 807.3 mg/kg bw/day ♀ 1111.2 mg/kg bw/day No treatment related adverse effects observed at the highest tested dose level	Crl:CD-1 (ICR) mouse Purity: 93.4%	Beck, W.; 2011b
Dog, 90-days (oral) (OECD 409)	0, 4000, 12000, 40000 ppm equivalent to: ♂: 0, 90.9, 267.8, 933.1 mg/kg bw/day ♀: 0, 102.7, 304.4, 820.4 mg/kg bw/day <u>NOAEL</u>: 4000 ppm ♂ 90.9 mg/kg bw/day ♀ 102.7 mg/kg bw/day <u>Effects at LOAEL</u>: ↑ liver weight Pigmentation of the liver Centrilobular degeneration ↑ alkaline phosphatase levels	Beagle dogs Purity: 93.4%	Beck, W.; 2012d

Dog, 1-year (oral) (OECD 452)	0, 200, 800, 4000, 8000 ppm equivalent to: ♂: 0, 4.3, 19.2, 92.0, 180.7 mg/kg bw/day ♀: 0, 4.5, 20.4, 92.0, 225.7 mg/kg bw/day <u>NOAEL</u>: ♂ 800 ppm (19.2 mg/kg bw/day) ♀ 4000 ppm (92.0 mg/kg bw/day) <u>Effects at LOAEL</u>: ♂: hepatocyte hypertrophy, pigmentation, ↑ alkaline phosphatase levels ♀: ↑ rel liver weight, hepatocyte hypertrophy, Pigmentation, ↑ alkaline phosphatase levels	Beagle dogs Purity: 93.4%	Beck, W.; 2012a
Rat, 28-days (dermal) (OECD 410)	0, 100, 300, 1000 mg/kg bw/d <u>NOAEL</u>: 1000 mg/kg bw/d No treatment related effects observed at the highest tested dose level	Slc: Wistar rats Purity: 93.4%	Ogata, H.; 2011
Rat oral via diet, 104 weeks (OECD 453)	0, 400, 2000, 7000, 15000 ppm equivalent to 0, 21.0, 105.1, 375.6 and 804.3 mg/kg bw/day (males) and 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw/d (females) <u>NOAEL</u>: 105.1 mg/kg bw/d (males) 26.7 mg/kg bw/d (females) <u>Effects</u>: ↓ body weight and bw gain (♂ at 15000 ppm, ♀at ≥ 2000 ppm) ↑ liver weight (♂ at ≥ 7000 ppm, ♀at ≥ 2000 ppm) ↑ hepatocellular hypertrophy (♂ at ≥ 7000 ppm, ♀at ≥ 2000 ppm) ↑ hepatocyte vacuolation (≥ 7000 ppm) ↑ total cholesterol (♂ at 15000 ppm, ♀at ≥ 7000 ppm) ↑ GGT (at 15000 ppm)	Crl:WI(Han) rats Purity: 93.4%	Beck, W.; 2012b
Mouse oral via diet, 78 weeks (OECD 451)	0, 700, 2000, 7000 ppm equivalent to 0, 82.5, 238.8 and 823.9 mg/kg bw/d in males	Crl:CD1(ICR) mice Purity: 93.4%	Beck, W.; 2012c

	and 0, 99.2, 280.3 and 994.0 mg/kg bw/d in females NOAEL: 823.9 mg/kg bw/d (males) 994.0 mg/kg bw/d (females) Effects: No adverse effects of treatment at the highest dose tested		

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

Rat:

13 week feeding study

Reference:	S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Rat.
Author(s), year:	Beck, W.; 2011a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0025
Guideline(s):	OECD 408 (1998), Directive 96/54/EC B.26 with Additional Testing for Neurotoxicity, EPA OPPTS 870.3100, and Japanese MAFF 12 Nousan 8147 (2000)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	Minor deviations not considered to affect the validity of the study.
Validity:	Yes

Material and methods:

Test Material: S-2200 Technical Grade (S-2200 TG)
- Lot/Batch: ST-0811G
- Purity: 93.4%
- Stability of test compound: Expiry date after completion of treatment; stability in diet checked in preliminary study (Covance study number 0333/289)
- Vehicle: None. Test material was mixed directly into diet.

Test animals:
- Species: Rat
- Strain: Wistar; Crl:WI(Han)
- Age: 7 weeks at start of treatment
- Weight at dosing: 170.1-214.7 g for males and 127.5-179.2 g for females
- Source: Charles River UK Ltd, Margate, UK
- Diet: SQC Rat and Mouse Maintenance Diet No1, Ground Fine *ad libitum*

The purpose of this study was to assess cumulative toxicity of mandestrobin when administered to rats in their diets for a period of 13 weeks. The results of the study should indicate potential target organs and should identify chemicals with neurotoxic potential.

Animal assignment and treatment:
Twelve rats per sex were allocated to each of 5 groups, and given mandestrobin in the diet at dietary concentrations of 0 (control), 800, 4000, 10000 and 20000 ppm. Doses were selected based on a prior three month feeding study (Sumitomo Chemical Co., Ltd., Osaka, Japan, Study number S1370).

Diet preparation, analysis and administration:
Dietary formulations were prepared weekly and the stability of the test article in diet was determined at 800 and 20000 ppm from a trial preparation prepared before the start of this study in Covance Study Number 0333/289. Achieved concentration and homogeneity analyses, for all concentrations of the dietary formulations, were performed in Weeks 1, 5 and 13 and were considered acceptable as the mean concentrations were between 90 and 110% of the target concentration.

Clinical observations:
All animals were observed at the beginning and the end of the working day to ensure they were in good health and displaying no signs of overt toxicity. Each animal was given a detailed physical examination at weekly intervals. An individual record was maintained of the clinical condition of each animal.

Food consumption and body weight:
Individual body weights were recorded on day -7, before treatment on the first day of dosing, at weekly intervals thereafter and before necropsy.
The amount of food consumed by each cage of animals was measured once weekly from Week -1. For each cage of animals, consumption was calculated as g/animal/week and the average daily consumption over the entire treatment period (g/animal/day) was calculated for each group. Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Ophthalmoscopic observation:
Investigations were performed on all animals pre-treatment and on control (Group 1), and high dose (Group 5) animals in Week 12. A mydriatic agent was instilled into the eyes before examinations.

Functional Observation Battery:
All animals were subjected to a battery of behavioural tests and observations before initiation of treatment and at weekly intervals thereafter.
In Week 13, an assessment was made of sensory reactivity to stimuli, grip strength and motor activity.
Observations (weekly):
Before removal from the home cage, each animal was observed and evaluated for the following: Posture, Activity, Gait, Arousal upon opening cage, Convulsion, Excessive vocalisation, Tremor.
After removal from the home cage, each animal was observed for the following: Ease of removal, Ease of handling, Excessive vocalisation, Tremor, Convulsion, Palpebral closure, Exophthalmus, Lacrimation, Lacrimation type, Salivation, Respiration, Piloerection, Appearance of fur.
Open field observations performed weekly: Each animal was placed into an open field arena for 2

minutes and the following observations were recorded: Latency to first step, Posture, Arousal, Circling, Gait type, Gait type severity, Stereotypy, Tremor, Convulsion.
The number of rears, faecal boli and urine pools, faecal consistency and the presence of polyuria during this 2-minute period were also recorded.
Open field (Week 13 only): Approach response, touch response, tail pinch, air righting ability, pupillary response, corneal tactile reflex test, auditory startle response, hindlimb foot splay, forelimb and hindlimb grip strength.
Locomotor activity (Week 13 only): Locomotor activity of each animal was assessed in an automated photocell activity recorder for 30 minutes. Activity counts were recorded at 2 minute intervals.

Haematology and clinical chemistry:
Blood samples (nominally 2 x 0.5 mL) were withdrawn from all animals in Week 13, two days after completion of the functional observational battery investigations. Samples were collected from the lateral caudal vein after an overnight period without food.
Due to a number of clotted samples or samples of insufficient volume at the scheduled bleed it was necessary to re-sample several animals at necropsy in order to measure all required parameters. These samples were obtained from the abdominal aorta. The re-sampled data were excluded from the calculation of group mean, standard deviation and statistical analyses.
The following parameters were determined for blood taken into EDTA anticoagulant: haemoglobin concentration (Hb), packed cell volume (PCV), mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), total and differential white blood cell count, red blood cell count, absolute reticulocytes, mean cell haemoglobin (MCH), and platelet count.
In addition, the following parameters were determined on plasma derived from whole blood taken into trisodium citrate anticoagulant: prothrombin time, activated partial thromboplastin time.
Blood smears were routinely prepared from all blood samples taken for haematology appraisal. Manual examination was not undertaken on these samples. Bone marrow smears were prepared at necropsy. They were fixed in methanol but not examined.
Clinical chemistry:
The following parameters were determined on plasma derived from whole blood collected into lithium heparin anticoagulant: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), sodium (Na), potassium (K), calcium (Ca), inorganic phosphorus (P), chloride (Cl), total protein, albumin, globulin, albumin/globulin ratio total cholesterol, glucose, urea, total bilirubin, creatinine, triglycerides.

Urine analysis:
Urine samples were collected from the first six numbered males and females in each group in Week 12. Samples were collected over a six-hour daytime period. Food and water were removed during collection. The urine collected from animal 99F (Group 4) in Week 12 (day 83) was of insufficient volume to perform all required assays. Therefore, repeat sampling was performed during Week 13 (day 85).
The following parameters were determined:
Colour, turbidity, microscopy of sediment volume, specific gravity, pH, protein, glucose, ketones, bilirubin, blood, urobilinogen.

Sacrifice and pathology:
All animals, including the one decedent (animal number 92, found dead day 33), were subjected to necropsy. Males were killed on day 95 and females were killed on day 96.
The scheduled necropsies were performed after overnight fasting. Following anaesthesia by sodium pentobarbitone, the animals were exsanguinated by the severing of major blood vessels.

A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded.

All tissues in the list below from all animals were preserved in the appropriate fixative.

adrenals (†) (š)
aorta (š)
bone marrow smear (femur) (a)
brain (e) (†) (š)
caecum (š)
colon (š)
duodenum (š) (f)
eyes (b) (š)
femur with bone marrow and articular surface (š)
gross lesions (š)
harderian glands (š)
heart (†) (š)
ileum (š)
jejunum (š)
kidney (†) (š)
larynx (š)
liver (†) (š)
lungs with mainstem bronchi and bronchioles (š)
mammary (š)
mandibular lymph nodes (š)
mesenteric lymph nodes (š)
muscle (quadriceps) (š)
nasal turbinates (š)
nasopharynx / nares (š)
oesophagus (š)
optic nerves (š)
ovaries (†) (š)
pancreas (š)
Peyer's patches (š)
Pituitary (š)
prostate (š)
rectum (š)
salivary glands–mandibular-sublingual-parotid (š)
sciatic nerves (š)
seminal vesicles (š)
skin (š)
spinal cord cervical (š)
spinal cord lumbar (š)
spinal cord thoracic (š)
spleen (†) (š)
sternum with bone marrow (š)
stomach (š)
testes + epididymides (c) (†) (š)
thymus (†) (š)
thyroids + parathyroids (š)
tongue (š)
trachea (š)
urinary bladder (š)
uterus (†) (š)
vagina (š)

(†) organs were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together
(š) tissues from the control and high dose and from the decedent animals were embedded, sectioned, H&E stained, and examined microscopically by the study pathologist.
Fixative = neutral-buffered 10% formalin except where indicated by:

a – methanol
b - Davidson's fluid
c Bouin's fixative

e - cerebrum, mid-brain and cerebellum/medulla
f - proximal region to include pyloric-duodenal junction

In addition, liver, spleen, thyroid, kidney (males), and gross lesions in the intermediate dose groups were microscopically examined by the study pathologist.

In addition, immunohistochemistry slides for the kidney for $\alpha_{2\mu}$globulin from two control (3M and 9M) and two high dose males (49M and 59M) were examined.

Statistical analysis:

ANOVA and Levene's tests were used to screen if data qualified (on the basis of heterogeneity) for parametric analysis using Dunnett's test. Statistical significance reported for Hb, MCH, MCHC, neutrophils (N), AST, ALT, Na, K, Ca, total protein, albumin, total cholesterol, creatinine are the results of pairwise comparison by Dunnett's test. Where data were too heterogeneous for parametric testing, or contained values of "0", non-parametric analysis was applied (Kruskal-Wallis, Terpstra-Jonckheere, and Wilcoxon). Statistical results for monocytes (M), bilirubin and gamma-glutamyltransferase are Wilcoxon pairwise comparisons.

Although a trend test was performed, no-statistically-significant difference in pairwise comparison was basically considered to negate a positive trend test.

Findings:

Clinical signs and mortality:
There were no clinical signs noted that were considered to be directly attributable to S-2200 TG.
There were no post-dosing observations.
There was one female decedent from the 4000 ppm dose group (animal 92F). Cause of death was not established, but there were no microscopic findings suggesting death was due to effects of S-2200 TG.

Body weight, body weight gain, and food consumption:
Males given 20000 ppm gained notably less weight (approximately 15%) over the duration of the treatment period than control. Suppression of absolute body weight (approximately 9% less than control) was also observed at the end of treatment for males given 20000 ppm.
In females, statistical significances from control in absolute body weight were only observed at some of the collection points. At the end of treatment, the body weight difference for females given 20000 ppm to controls was approximately 4%. Suppression of body weight gain, although not statistically significant, was also observed at 20000 ppm (approximately 11%).
There was inter-group variation in weekly food consumption, but there was no consistent pattern in the data to indicate an effect of treatment.

Table 17: Mean compound intake, body weight, body weight gain, and food consumption

	Males					**Females**				
Diet concentration (ppm)	**0**	**800**	**4000**	**10000**	**20000**	**0**	**800**	**4000**	**10000**	**20000**
Mean substance intake (mg test material/kg bw/day, week 1-13)	-	54	282.6	742.7	1544.6	-	61.6	320.1	788.5	1886.5
Group mean body weights (in g, week 13)	383.4	397.4	381.2	373.8	349.9	233.7	225.0	225.6	231.6	223.5
Group mean body weight gains (in g, start to week 13)	188.1	204.1	193.0	183.9	160.2	83.1	79.3	78.6	85.5	74.2
Mean food consumption (g/animal/day, week 1-13)	22.3	22.7	22.9	23.2	23.0	17.4	16.5	17.3	17.0	19.5

Ophthalmoloscopy:
There were no treatment-related pathological findings in the ophthalmoscopical examinations.

Functional Observation Battery data:
There were no overt effects of treatment or differences from control in the indices of:
Posture, activity, gait, tremors, convulsions, excessive vocalisations, arousal, ease of removal or handling, palpebral closure, exophthalmus, lacrimation, type of lacrimation, salivation, respiration, piloerection, appearance of fur, or other changes.
In the open field observations, there were no effects on latency to first step, posture, arousal, circling, gait type/severity, stereotypy, tremors, convulsions, number of rears, number of faecal boli, number of urine pools, faecal consistency, or polyuria.
There were no overt effects on the approach response, touch response, tail pinch, air righting, pupillary response, corneal tactile response, auditory response, hindlimb foot splay, or hindlimb grip strength, or locomotor activity.
Forelimb grip strength was statistically significantly reduced in male animals in the highest dose group. In the absence of any other effect, this isolated finding in one gender was considered incidental.

Table 18: Functional observation battery data: Forelimb and hindlimb grip strength

	Males					**Females**				
Diet concentration (ppm)	**0**	**800**	**4000**	**10000**	**20000**	**0**	**800**	**4000**	**10000**	**20000**
Mean forelimb grip strength (kg) (SD)	1.023 (0.270)	1.008 (0.253)	1.052 (0.292)	1.070 (0.149)	**0.760*** (0.235)	0.751 (0.207)	0.787 (0.178)	0.707 (0.205)	0.757 (0.264)	0.742 (0.233)
Mean hindlimb grip strength (kg) (SD)	0.613 (0.126)	0.642 (0.191)	0.625 (0.178)	0.732 (0.132)	0.665 (0.118)	0.620 (0.124)	0.612 (0.157)	0.607 (0.161)	0.620 (0.117)	0.633 (0.373)

*p < 0.05 (ANOVA)

Haematology and clinical chemistry:
Some changes in haematological parameters reached statistical significance in the highest dose group. The changes were within the ranges of historical control data.
Historical control data was available from studies performed between March 2005 and January 2008.
For haematology and clinical chemistry, 15 different studies were available (203 and 223 animals). For organ weights, 16 different studies with 163 analysed animals were available.

Table 19: Haematological findings

	Males						**Females**					
Diet conc. (ppm)	**0**	**800**	**4000**	**10000**	**20000**	**HCD 95% range**	**0**	**800**	**4000**	**10000**	**20000**	**HCD 95% range**
Hb (g/dl)	16.7	16.5	16.5	16.6	**16.2****	14.5-18.0	15.7	15.7	15.3	15.7	**15.0***	14.2-17.0
MCH (pg)	18.2	18.1	18.0	17.9	**17.5***	16.6–19.8	18.8	18.4	18.6	18.8	18.4	
MCHC (g/dL)	35.5	35.4	34.9	35.1	**34.6***	31.5–36.2	34.9	34.3	34.5	34.0	34.2	

Neutrophils (10^9/L)	1.3	1.0	1.1	1.2	**0.9****	0.5–3.0	0.6	0.5	0.6	0.6	0.6	
Monocytes (10^9/L)	0.2	0.2	0.2	0.1	**0.1***	0.0–0.3	0.1	0.1	0.1	0.1	0.1	
Eosinophils (10^9/L)	0.2	0.2	0.2	0.1	0.1		0.1	0.1	0.1	0.1	0.1	
PLT (10^9/L)	891	953	914	1000	1014	688-1211	932	865	903	974	1004	
PT (s)	20.9	21.6	20.7	20.2	21.2		20.5	21.2	20.6	19.6	19.5	17.6-21.8
APTT (s)	24.1	21.2	23.3	28.5	27.6	16.0-26.5	20.5	20.2	21.7	22.1	22.7	14.0-23.5

Hb Haemoglobin concentration
MCH mean cell haemoglobin
MCHC mean cell haemoglobin concentration
PLT platelets
PT prothrombin time
APTT activated partial thromboplastin time
HCD historical control data (95% interval)
* $p < 0.05$
** $p < 0.01$

The changes in clinical chemistry parameters were mild and within the range of historical control data (if available).
A dose dependent increase in cholesterol levels was observed in both genders that reached statistical significance at 10000 and 20000 ppm.

Table 20: Clinical chemistry parameters

	Males						**Females**					
Diet conc. (ppm)	**0**	**800**	**4000**	**10000**	**20000**	**HCD**	**0**	**800**	**4000**	**10000**	**20000**	**HCD**
AST (IU/L)	75	**65***	66	69	**62****	50–87	68	65	64	62	**56****	49–103
ALT (IU/L)	46	**33***	**32***	39	**32***	26–69	29	29	32	30	28	
ALP (IU/L)	66	66	66	66	66		34	35	30	33	30	
γGT (IU/L)	2	2	2	2	**5****		2	2	2	3	**4****	
Total Protein (g/L)	69	70	70	71	71		71	70	70	74	**77****	63–78
Albumin (g/L)	45	45	46	47	47		48	49	47	50	**51***	34–56
Globulin (g/L)	24	25	24	24	24		24	21	23	24	25	

Albumin/ Globulin ratio	1.9	1.8	1.9	2.0	2.0		2.0	2.3	2.1	2.1	2.0	
Total Bili rubin (µmol/L)	2.1	2.0	**1.5***	**1.4***	**1.4****	0.5–2.9	2.1	1.8	**1.4****	2.0	2.2	1.0–3.5
Na (mmol/L)	145	**144***	144	**144***	145	132–148	144	143	143	143	144	
K (mmol/L)	4.7	4.8	4.7	4.7	4.9		4.1	4.1	4.1	**4.7****	**4.7****	3.5–5.0
Ca (mmol/L)	2.71	2.67	2.66	2.71	2.74		2.73	2.71	2.71	**2.85****	**2.88*****	2.54–2.91
Cl (mmol/L)	103	103	102	102	101		103	104	103	102	103	
Total chole sterol (mmol/L)	2.0	2.2	2.3	**2.6****	**2.8*****		1.5	1.7	1.8	**2.5*****	**2.6*****	
Creatin-ine (µmol/L)	37	35	35	36	36		40	40	38	40	**34****	26–51
Triglyceri des (mmol/L)	0.96	0.93	0.81	0.83	0.64		0.73	0.78	0.71	0.7	1.17	

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Urine analysis:
There were no differences between control and any treated group/sex in urine volume and specific gravity.

Sacrifice and pathology:
Organ weight increases were noted in the liver of treated males and females. Mean unadjusted and relative liver weights were outside historical control ranges for animals of this strain and age housed at this laboratory in males given ≥ 4000 ppm and females given ≥ 10000 ppm.

Large liver was recorded among males given 4000, 10000 or 20000 ppm and females given 20000 ppm. Mottled appearance was also recorded for the liver of males given 4000, 10000 or 20000 ppm. These changes generally correlated with findings seen microscopically.

Historical control data was available from studies performed between March 2005 and January 2008. For organ weights, 16 different studies with 163 analysed animals were available.

A slight increase in relative kidney weight was observed in males at the highest dose group.

In the thyroid gland, increased follicular cell hypertrophy was observed from 4000 ppm onwards in both sexes.

No significant weight changes or other gross pathological lesions attributable to treatment were detected in other organs.

Microscopic findings in controls were generally infrequent, of a minor nature and consistent with the usual pattern of findings in animals of this strain and age.

Treatment related findings were seen in the liver, thyroid and kidney.

In the liver, hepatocyte hypertrophy was recorded for males and females given 4000, 10000 or 20000 ppm, with incidence and severity increasing with dose, and correlated with large and/or mottled liver. Hepatocyte hypertrophy was characterised by hepatocytes with increased levels of pale, slightly granular cytoplasm. In the most severely affected livers, the hypertrophy involved hepatocytes in all zones of the liver. In less severely affected livers, there was no clear zonal distribution of the enlarged hepatocytes.

The agonal congestion/haemorrhage recorded for some treated males was considered to be a consequence of the liver hypertrophy by the study authors.

Table 21: Organ weights and histopathology

	Males					**Females**				
Diet concentration (ppm)	**0**	**800**	**4000**	**10000**	**20000**	**0**	**800**	**4000**	**10000**	**20000**
Liver										
Organ weight (absolute and relative)										
Absolute weight (g)	8.36	9.03	**9.50*** **	**10.18*** **	**12.02*** **	5.80	5.53	6.08	**6.79** **	**8.34*** **
% change		7.9	13.6	21.8	43.8		-4.7	4.8	17.0	43.8
Relative weight (Ratio %)	2.26	2.37	**2.61*** **	**2.89*** **	**3.54*** **	2.55	2.51	2.76	**3.05*** **	**3.81*** **
% change		4.9	15.5	27.9	56.6		-1.6	8.2	19.6	49.4
Macroscopic pathology										
No. examined	12	12	12	12	12	12	12	11	12	12
Large	0	0	2	7	10	0	0	0	0	4
mottled	0	0	1	3	5	0	0	0	0	0
Histopathology: hepatocyte hypertrophy										
No examined	12	12	12	12	12	12	12	11	12	12
Grade -	12	12	0	0	0	12	12	7	3	0
Grade 1	0	0	5	0	0	0	0	4	6	0
Grade 2	0	0	7	0	0	0	0	0	3	0
Grade 3	0	0	0	7	0	0	0	0	0	0
Grade 4	0	0	0	5	1	0	0	0	0	3
Grade 5	0	0	0	0	11	0	0	0	0	9
Agonal congestion/haemorrhage										

P	0	0	1	3	5	2	0	0	0	0
Thyroid										
Follicular cell hypertrophy										
No. examined	12	12	12	12	12	12	12	11	12	12
Grade -	10	10	6	3	5	10	11	7	7	6
Grade 1	2	2	6	8	5	2	1	4	5	6
Grade 2	0	0	0	1	2	0	0	0	0	0
Kidney										
Organ weight (absolute and relative)										
weight (g)	1.934	1.948	1.988	1.923	1.897	1.363	1.252	1.275	1.277	1.288
Relative weight (Ratio %)	0.524	0.512	0.546	0.545	0.560	0.599	0.569	0.578	0.575	0.589
Hyaline droplets										
No. examined	12	12	12	12	12	12	0	0	0	12
Grade -	2	3	1	1	0	12	0	0	0	12
Grade 1	6	6	6	3	2	0	0	0	0	0
Grade 2	4	3	4	7	8	0	0	0	0	0
Grade 3	0	0	1	1	2	0	0	0	0	0
α2u globulin positive reaction by immunohistochemistry										
No. examined	2	0	0	0	2	0	0	0	0	0
Grade -	0	0	0	0	0	0	0	0	0	0
Grade 1	1	0	0	0	0	0	0	0	0	0
Grade 2	1	0	0	0	0	0	0	0	0	0
Grade 3	0	0	0	0	2	0	0	0	0	0

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
Grade 1 = minimal, Grade 2 = slight, Grade 3 = moderate, Grade 4 = moderately severe, Grade 5 = severe

Conclusion:

In the original study report, the study authors set the NOAEL at 10000 ppm. Increases in liver weight, hepatocellular hypertrophy, and follicular cell hypertrophy in the thyroid gland observed from 4000 ppm were considered to be secondary to liver enzyme induction and increased catabolism of thyroid hormones in a manner similar to the observed effects induced by phenobarbital (position paper Yamada 2012a and study Asano 2012e).
The hyaline droplets (increased incidence from 4000 ppm onwards) in the kidneys of male rats contained α2μ-globulin and were thus considered to be not relevant for human risk assessment.

Evidence for a phenobarbital-like mode of action was considered insufficient in the EFSA peer

review. Thus, the NOAEL for male rats is revised to 800 ppm (54 mg/kg bw/day) based on absolute (13.6%) and relative (15.5%) liver weight increase and hepatocellular hypertrophy at 4000 ppm. The NOAEL for female rats was set at 4000 ppm (320.1 mg/kg bw/day) based on liver weight increase (17% absolute and 19.6% relative increase), hepatocellular hypertrophy, and thyroid follicular cell hypertrophy, increased cholesterol levels at 10000 ppm

104 weeks feeding study – summary

(for details see *section 4.10.1.1 Carcinogenicity: oral*)

Groups of 70 male and 70 female Wistar rats were offered mandestrobin in the diet at concentrations of 0 (control), 400, 2000, 7000, 15000 ppm. After 52 weeks, satellite groups of 20 males and 20 females were used for interim sacrifice and the remaining survivors sacrificed after 104 weeks of treatment.

Due to the magnitudes of the decreased body weight and body weight gain (at 15000 ppm) and toxicological alterations in the liver including increased liver weights in combination with a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (both at ≥ 7000 ppm) and increased blood biochemistry parameters (total cholesterol and gamma-glutamyltransferase in males offered 15000 ppm), the NOAEL for males was considered to be 2000 ppm (105.1 mg/kg bw/day).

For females, body weight and body weight gain was significantly decreased at ≥ 2000 ppm following 104 weeks of treatment. Toxicological alterations in the liver in females included increased liver weights (at ≥ 2000 ppm), a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (at ≥ 2000 ppm and at ≥ 7000 ppm, respectively) and increased blood biochemistry parameters (increased total cholesterol and increased gamma glutamyltransferase at ≥ 7000 ppm and at ≥ 15000 ppm, respectively). Therefore, the NOAEL for females was considered to be 400 ppm (26.7 mg/kg bw/day) for this study following 104 weeks of treatment.

Regarding the carcinogenic potential of mandestrobin, no increase of neoplastic findings exceeding the historical control range was observed in any organ of treated animals, with exception of benign sex-cord stromal tumours in the ovary, which are discussed in detail in section *4.10 Carcinogenicity*.

Mouse:

13 week feeding study

Reference:	Amended Final Report 1: S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Mouse
Author(s), year:	Beck, W.; 2011b
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0023
Guideline(s):	OECD 408, Directive 2001/59/EC (Annex 5D) B.26, EPA OPPTS 870.3100, and Japanese MAFF 12 Nousan 8147 (2000), 2-1-9, 90 day repeated oral toxicity studies
GLP:	Yes (laboratory certified by National Authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	The stability of the test article in diet was confirmed before the start of this study under Covance Study Number 0333/289
Vehicle:	None. Test material was mixed directly into diet.

Test animals:

Species:	Mouse
Strain:	Crl:CD-1 (ICR)
Age:	approximately 7 weeks at start of treatment
Weight at dosing:	30.6 – 38.1 g (males); 22.2-28.9 g (females)
Source:	Charles River UK Ltd, Margate, UK
Diet:	Finely ground SQC Rat and Mouse Maintenance Diet No 1 (Special Diets Services Ltd, Witham, UK) *ad libitum*

Animal assignment and treatment:
Twelve mice per sex were allocated to each of four dose groups, and offered mandestrobin in the diet at fixed concentrations of 0 (control), 1750 ppm, 3500 ppm, and 7000 ppm for 13 weeks. Animals were housed in groups of three and were provided with environmental enrichment.

Table 22: Diet concentration and daily dose levels

	Males				**Females**			
Diet concentration (ppm)	**0**	**1750**	**3500**	**7000**	**0**	**1750**	**3500**	**7000**
mg test material/kg bw/day	-	204.1	404.9	807.3	-	251.8	529.1	1111.2

Diet preparation, analysis and administration:
The test article was formulated weekly as a diet mix in SQC Rat and Mouse Maintenance Diet No 1 (Ground Fine). Due to unused diet being discarded in error during Week 1, a new batch of each formulation (all groups) was prepared on day 4, in order to provide sufficient diet to complete Week 1.
Duplicate samples were taken from the top middle and bottom of the mixer after diet preparation on week 1, and week 13 of the dosing period, and achieved concentrations were between 90 and 110% of the target concentration.

Clinical observations:
All animals were observed at the beginning and the end of the working day to ensure they were in good health.
All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a detailed physical examination at weekly intervals. An individual record was maintained of the clinical condition of each animal.

Food consumption and body weight:
Individual body weights were recorded on day -7, before treatment on the first day of dosing, at weekly intervals thereafter, and before necropsy. In addition, body weights were inadvertently recorded for all animals on day 4. These data are maintained within the raw data but not reported.
The amount of food consumed by each cage of animals was measured once weekly from Week -1.
Consumption was calculated as g/animal/week and as average daily consumption over the entire treatment period (g/animal/day).
Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Haematology and clinical chemistry:
Blood samples were withdrawn in Week 13 from non-fasted, anaesthesised animals. Samples from the first six numbered males and females in each group were analysed for haematology parameters and blood taken from the last six numbered animals were analysed for blood biochemistry parameters. Due to samples being clotted or of insufficient volume, it was necessary to re-sample animals 14 (Group 2M), 30 (Group 4M) and 65 (Group 2F) at necropsy.
The following parameters were determined on blood taken into EDTA anticoagulant: red blood cell count, absolute reticulocytes, packed cell volume (PCV), mean cell volume (MCV), haemoglobin concentration (Hb), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelet count (PLT), and total and differential white blood cell count. Blood smears were routinely prepared from all haematology samples. Manual examination was not undertaken on these samples.
The following parameters were determined on plasma derived from whole blood collected into heparin anticoagulant: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, sodium, potassium, calcium, inorganic phosphorus, chloride, total protein, albumin, globulin, albumin/globulin ratio, total cholesterol, glucose, urea, total bilirubin, creatinine, triglycerides.

Urine analysis:
Not performed.

Sacrifice and pathology:
All animals were subjected to necropsy.
A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded.

All tissues in the tissue list below from all animals were preserved in the appropriate fixative.

adrenals (†) (š)
aorta (š)
brain (†) (š)
caecum (š)
colon (š)
duodenum (š)
eyes (š)
femur with bone marrow and stifle joint (š)
gall bladder (š)
gross lesions (š)
ovaries (†) (š)
pancreas (š)
Peyer's patch (š)
pituitary (š)
prostate (š)
rectum (š)
salivary glands – mandibular, sublingual, parotid (š)
sciatic nerves (š)
seminal vesicles (š)
skin and subcutaneous tissue (š)

harderian glands (š)
heart (†) (š)
ileum (š)
jejunum (š)
kidney (†) (š)
larynx (š)
liver (†) (š)
lungs (š)
mammary (š)
mandibular lymph nodes (š)
mesenteric lymph nodes (š)
muscle (quadriceps) (š)
nasal cavity (š)
nasopharynx / nares (š)
oesophagus (š)
optic nerves (š)
spinal cord cervical (š)
spinal cord lumbar (š)
spinal cord thoracic (š)
spleen (†) (š)
sternum with bone marrow (š)
stomach (š)
testes + epididymides (e) (†) (š)
thymus (†) (š)
thyroids + parathyroids (š)
tongue (š)
trachea (š)
urinary bladder (š)
uterus including cervix (†) (š)
vagina (š)
zymbal glands (g)

Organ weights
Animals were weighed before necropsy. The organs denoted by (†) from all animals in the tissue list were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together.

Histology
Gross lesions from all animals and tissues denoted by (š) in the tissue list from control (Group 1) and high dose (Group 4) animals were embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Liver, spleen, kidney, thyroid and caecum from all animals in Groups 2 and 3 were processed to slide stage.
Additional tissues were also included to complete standard tissue blocks.

Pathology
Gross lesions from all animals and tissues denoted by (Š) in the tissue list from control and high dose animals were examined microscopically by the Study Pathologist. In addition, the liver and thyroid from all low and intermediate dose (Groups 2 and 3, respectively) animals were examined.

Statistics:
Absolute body weights, body weight gains, necropsy body weights, organ weights and organ to necropsy body weight ratios were analysed using one-way analysis of variance (ANOVA), separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.
Food consumption, haematology and blood biochemistry variables were analysed using two-way analysis of variance (ANOVA). Levene's test for equality of variances across groups, between sexes and for any interaction was performed and where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex separately, a linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of a sex effect only ($p < 0.01$), the data were analysed using one-way ANOVA for each sex separately.
Non-parametric analyses were performed for clinical pathology parameters with values above or below the limit of the assay and for Monocytes, Eosinophils, Basophils and Large Unstained Cells. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.
Macroscopic and microscopic findings data were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk.
For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.
For microscopic findings, comparisons were made between the incidence in each of the treated groups and that in the control group. Where a given finding had more than two categories, the Wilcoxon-Mann-Whitney rank sum test was performed; where only two categories were observed, Fisher's Exact test was used.

Findings:

Clinical signs and mortality:
All animals survived to the scheduled sacrifice. There were no clinical signs noted that were considered to be directly attributable to S-2200 TG. There were no post-dosing observations.

Food consumption:
Over the entire treatment period, there were no notable effects on food consumption (see Table 23).

Body weight and body weight gain:
There were no statistically significant effects on body weight or body weight gain for males.
In females, when compared to controls, mean weight gain was lower in the treated groups (90%, 89% and 73% of controls for animals offered 1750, 3500 and 7000 ppm, respectively) with differences for animals offered 7000 ppm being statistically significant at the $p < 0.05$ level.
The animals did not show signs of systemic toxicity.

Table 23: Food consumption, body weight and body weight gain

	Males				**Females**			
Diet concentration (ppm)	**0**	**1750**	**3500**	**7000**	**0**	**1750**	**3500**	**7000**
Food consumption, mean weeks 0-13 (g/mouse/day)	5.2	5.0	4.9	5.0	4.6	4.5	4.8	4.9
Terminal bodyweight (g)	43.6	43.7	42.7	44.4	33.5	32.3	32.1	30.7
Bodyweight gain, weeks 0-13 (g)	10.5	9.6	8.9	9.7	8.3	7.5	7.4	**6.1***

Level of statistical significance: * $p < 0.05$

Haematology and clinical chemistry:
S-2200 TG had no obvious or adverse effects on haematology parameters.

Statistically significant decreases in haemoglobin concentration (Hb) and packed cell volume (PCV) were seen in males offered 3500 ppm. In the absence of a dose response and mean values being within historical control ranges, these changes are considered to be incidental.
Statistically significant increased platelet counts (PLT) in males offered 1750 or 7000 ppm were considered not to be treatment related, since the increased mean platelet values were not dose-responsive and the value at 7000 ppm was within the historical control range.
The significant dose-responsive changes seen for mean cell haemoglobin concentration (MCHC) were considered not to be treatment related as the differences observed were small, and none of the pairwise comparisons were significant.
S-2200 TG had no obvious or adverse effects on blood biochemistry test results. The following statistically significant difference between control and treated female animals was observed:
The apparent statistically significant decrease (15.7%) in mean glucose for females given 7000 ppm was considered not to be toxicologically significant, since the value at 7000 ppm was within the historical control range.
The significant dose-responsive changes in chloride (Cl), urea (UREA) and total bilirubin (T BILI) were considered not to be treatment related, since none of the pairwise comparisons were significant.

Table 24: Haematology and clinical chemistry parameters

	Males					**Females**				
Diet concentration (ppm)	**0**	**1750**	**3500**	**7000**	**HCR**	**0**	**1750**	**3500**	**7000**	**HCR**
	Haematology									
Hb (g/dL)	14.6	14.3	**13.2 ****	14.1	12.0-15.1	14.6	14.9	14.8	14.1	-
PCV (%)	44.8	44.6	**41.6 ***	44.9	38.0-50.4	45.1	45.8	45.8	44.3	-
PLT (10^9/L)	1292	**1756 ****	1450	**1666 ***	941-1721	1555	1351	1390	1526	-
MCHV (g/dL)	32.3	32.1	31.7	31.5	-	32.4	32.5	32.3	31.9	-
	Clinical pathology									
GLUC (mmol/L)	8.9	9.7	9.3	9.1	7.9-12.0	10.8	10.3	9.3	**9.1***	-
Cl (mmol/L)	109	109	109	107	-	112	112	112	112	-
Urea (mmol/L)	6.4	6.3	6.7	7.3	-	7.1	6.8	6.6	6.6	-
T Bili (µmol/L)	2.5	1.8	2.1	1.8	-	1.7	1.7	1.7	1.9	-

* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. HCR : Historical Control Range

Sacrifice and pathology:

Organ weight increases were noted in the liver of treated males and females.

Mean unadjusted and relative liver weights were increased in all treated groups (both sexes) and, in the majority of cases, were outside historical control ranges for animals of this strain and age housed at this laboratory.

Although there were no histopathological findings in the liver, this finding is considered to be an adaptive change related to drug metabolism and not an adverse toxic effect. The mode of action for the liver weight increase is considered to be due to liver enzyme induction (CYP2B), via activation of the constitutive androstane receptor by S-2200 TG, as evidenced by mode of action work. Because an adaptive mechanism is ascribable for the increase in liver weights rather than a pathological effect, the increases may be considered non-adverse, particularly in the absence of

any biochemical or histological markers of liver pathology. A statistically significant increase in relative to body (24%) spleen weight was seen in males offered 3500 ppm, when compared to control. However, in the absence of a dose response, these changes are considered not to be treatment related.

Statistically significant differences between the control and treated animals for all other organs were considered incidental as they reflect normal individual variation.

Weights of all other organs were not affected by treatment.

Most tissues were macroscopically and microscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in mice of this strain and age.

There were no macroscopic and microscopic findings due to effects of mandestrobin.

Table 25: Organ weights and histopathology of the liver

	Males				**Females**			
Diet concentration (ppm)	**0**	**1750**	**3500**	**7000**	**0**	**1750**	**3500**	**7000**
Organ weights								
Liver weight (g)	2.00	2.11	2.14	**2.35*****	1.58	1.74	1.64	1.81
% change		5.5	6.9	17.4		10.1	3.9	14.5
Ratio (%) to body weight	4.55	4.73	**4.95****	**5.22*****	4.65	**5.20***	5.02	**5.66*****
% change		4.0	8.8	14.7		11.8	8.0	21.7
Histopathology								
Focal necrosis	2	1	4	5	3	2	6	3
Inflammatory cell foci	11	11	11	11	12	12	12	12
Mitotic figures	0	1	0	0	0	0	0	2
Hepatocyte vacuolation	0	0	0	0	0	1	0	0
Glycogen vacuolation	8	8	7	9	12	10	11	12
Pigmented histiocytes	0	2	0	0	0	1	0	1
Agonal congestion/ haemorrhage	0	0	1	0	0	2	0	0
Haemopoiesis	0	1	0	0	0	0	0	0

* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$

Conclusion:

In a 13 week dietary toxicity study in the mouse (0, 1750, 3500 and 7000 ppm S-2200 TG), liver weight increases were noted in all treated groups in both sexes. Because an adaptive mechanism is ascribable for the increase in liver weights rather than a pathological effect, the increases may

be considered non-adverse, particularly in the absence of any biochemical or histological markers of liver pathology.

In the absence of other treatment-related adverse findings, the No-Observed-Adverse-Effect-Level (NOAEL) is considered to be 7000 ppm (807.3 mg/kg bw/day for males and 1111.2 mg/kg bw/day for females, respectively).

78 weeks feeding study – summary

(for details see *section 4.10.1.1 Carcinogenicity: oral*)

Groups of 51 male and 51 female CD-1 mice were given mandestrobin in the diet at concentrations of 0 (controls), 700, 2000, and 7000 ppm for 78 weeks. Satellite groups of 12 mice per sex per dose were reared up to 52 weeks for interim sacrifice.

Treatment with mandestrobin was well-tolerated. In the absence of adverse effects, the NOAEL for this study was considered to be 7000 ppm (823.9 mg/kg bw/day for males and 994.0 mg/kg bw/day for females), the top dose tested, following 78 weeks of treatment.

Dog:

90-day feeding study

Reference:	Amended Final Report 2: S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Dog
Author(s), year:	Beck, W.; 2012d
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0024
Guideline(s):	OECD 409, US EPA OPPTS 870.3150, JMAFF 2-1-9, 90-day repeated oral toxicity studies
GLP:	Yes (Lab certified by National Authority)
Deviations:	Minor deviations not affecting the validity of the study
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch No.:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date stated as 21 November 2011 (after end of treatment)
Vehicle:	None
Test animals:	
Species:	Dog
Strain:	Beagle
Age:	20 – 24 weeks at initiation of dosing (males and females)
Weight at dosing:	Males: 10.21 – 12.01 kg; Females: 8.38 – 10.74 kg
Source:	Harlan, Bicester, UK.
Diet:	5L66 Certified High Density Canine Diet (IPS Product Supplies Ltd, London, UK); 300 g/day

Animal assignment and treatment:

Sixteen healthy animals per sex were selected based on the results of pre-test examinations. Animals were randomly distributed into 4 animals/sex/group based on the most recent body weight data, except for separation of familial animals.

Diet preparation, analysis and administration:

The test article was administered orally by incorporation into the diet. Each animal had *ad libitum* access to the dietary formulations for approximately 8 hours/day for at least 13 weeks. Control animals received untreated diet. The high dose was selected as a limit dose of approximately 1000 mg/kg bw/day.

Fresh batches of dietary formulation were prepared weekly. All diets were hand mixed with mains water into clumps of irregular size and shape. The formulations were stored at room temperature in the dark before administration.

Accuracy and homogeneity of preparation was confirmed by analysis. Homogeneity of mixing was determined at the top, middle, and bottom of each mixture. Samples were taken before treatment, and on weeks 1 and 13. Stability in diet for at least 9 days at ambient temperature was established before the study started.

Table 26: Diet concentration and daily dose levels

	Males				**Females**			
Diet concentration (ppm)	**0**	**4000**	**12000**	**40000**	**0**	**4000**	**12000**	**40000**
mg test material/kg bw/day	0	90.9	267.8	933.1	0	102.7	304.4	820.4

Clinical observations:

All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a daily detailed physical examination. Animals were observed approximately 1 and 4 hours after presentation of the diet. One control male (animal number 3) received topical and oral antibiotics for presumed bites.

All animals were subjected to a battery of behavioural tests and observations before initiation of treatment and at once weekly intervals thereafter. The unrestrained behaviour of each animal was observed in an examination room for approximately 1 to 2 minutes.

Observations included the following:

Level of consciousness
Head posture
Neck posture
Limb posture (standing)
Tremors
General level of activity
Head co-ordination
Circling
Gait
Unusual behavior

Visual fixating response test and auditory startle tests were assessed at the end of the observation period.

Following the unrestrained observations all animals were subjected to neurological examinations each week.

Examinations included the following:

Proprioceptive paw positioning	Righting postural reaction
Hemihopping postural reactions	Wheel barrowing postural reactions
Muscle tone	Palpebral closure
Nictitating membrane	Palpebral reflex
Eye position	Eye movement
Pupil size	Pupillary light reflex
Lacrimation	Salivation
General reactions	General clinical observations

Body weight, general clinical signs, heart rate and rectal temperatures were taken as part of the data set for assessment of neurobehavioral effects of test articles.

Food consumption and body weight:

Food consumption for all animals was measured daily from the time of allocation until termination of the dosing period.

Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Body weight was measured in all animals once weekly throughout the dosing period, from Week -3 until termination.

Ophthalmoscopic observation:

A mydriatic agent was instilled into the eyes before examinations. On day -2, a mydriatic agent was administered to all animals but no ophthalmic examination took place. Eyes of all animals were observed once pre-test (day -1) and at Week 12.

Haematology and Clinical Chemistry:

Blood was sampled from all animals before the start of treatment (Week -1) and at weeks 4, 8, and 13 of the administration period (sampling was performed after animals were fasted overnight). Samples were taken from jugular vein and collected into EDTA (for cell parameters), trisodium citrate (for clotting function), and lithium heparin (blood chemistry).

The blood samples collected into trisodium citrate anticoagulant from one male and three female in Week 8 (day 52) were unsuitable for analysis, so blood samples were recollected and re-examined on day 56. Additionally, the prothrombin time results from two females were estimated due to the analysis software having difficulties interpreting the data. The blood sample collected into lithium heparin anticoagulant in Week 13 (day 87) for one male was unsuitable for analysis; so sampling was repeated on day 92.

The following parameters were examined:

Haematology: Haemoglobin concentration (HGB), Red blood cell count (RBC), Mean cell haemoglobin (MCH), Mean cell haemoglobin concentration (MCHC), Mean cell volume (MCV), Packed cell volume (PCV), Platelet count (PLT), Total and differential white blood cell count, Reticulocytes, Prothrombin time (PT), and Activated partial thromboplastin time (APTT)

Blood Chemistry: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), γ-Glutamyl transferase (GGT), Sodium (Na), Potassium (K), Calcium (Ca), Inorganic phosphorus (IP), Chloride (Cl), Triglycerides (TG), Total protein (TP), Albumin (Alb), Globulin (G), Albumin-globulin ratio (A/G), Total cholesterol (Cho), Glucose (Glu), Urea (U), Total bilirubin (Bil), and Creatinine (Cre).

Urine analysis:

Urine samples were collected overnight from all animals before the start of treatment (Week -1) and in Weeks 3, 7, and 12 of the administration period; food and water were removed during collection. Due to spillage, urine volume was not measured for one male in Week 3.

The following parameters were examined: Urinary volume (Vol), Colour (Col), Turbidity, Specific Gravity (S.G.), pH, Protein (Pro), Glucose (Glu), Ketones (Ket), Urobilinogen (Uro), Bilirubin (Bil), Blood, and microscopic sediment.

Sacrifice and pathology:

All animals survived until scheduled sacrifice, and were sacrificed with sodium thiopentone, and exsanguinated from the major blood vessels.

The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, epididymides, thymus, thyroids (with parathyroids), and uterus (including cervix). Bilateral organs were weighed together.

Histopathological examination: After necropsy, the following organs and tissues collected from all animals were fixed in 10% neutral buffered formalin, except bone marrow smears which were fixed in methanol, testes and epididymides which were fixed in Bouin's solution and eyes/optic nerves fixed in Davidson's fluid.

Adrenals	Oesophagus
Aorta	Ovaries
Bone and marrow (sternum)	Oviducts
Brain	Pancreas
Caecum	Pituitary
Colon	Prostate
Duodenum	Rectum
Eyes and Optic nerves	Salivary gland – mandibular
Bone with marrow (femur) and articular surface	Salivary gland – Sublingual, parotid
Gallbladder	Sciatic nerves
Gross lesions	Skin and subcutaneous tissue
Heart	Spinal cord (cervical, thoracic, lumbar)

Ileum
Jejunum
Kidneys
Lacrimal glands
Larynx
Liver
Lungs with bronchi and bronchioles
Mammary glands
Mandibular lymph nodes
Mesenteric lymph nodes
Muscle
Nasal cavity
Nasopharynx
Spleen
Sternum with bone marrow
Stomach
Testes and epididymides
Thymus
Thyroids and parathyroids
Tongue
Trachea
Ureters
Urinary bladder
Uterus (including cervix)
Vagina

Statistical analysis:

Absolute body weights, body weight gains, necropsy body weights, food consumption, heart rates, body temperatures, haematology, blood biochemistry and urine analysis variables were analysed using two-way analysis of variance (ANOVA). Levene's test for equality of variances across groups, between sexes and for any interaction was performed. Where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex separately, a linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of group effects, sex effects or a sex by group interaction ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

The non-parametric analyses described above were also performed for clinical pathology parameters with values above or below the limit of the assay and for Monocytes, Eosinophils, Basophils and Large unstained cells.

Organ weights and organ to necropsy body weight ratios were analysed using oneway analysis of variance (ANOVA), separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods, as previously described.

Organ weights were also analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, for each sex separately, using the necropsy body weight as covariate. This analysis depends on the assumption that the relationship between the organ weights and the covariate is the same for all groups and the validity of this assumption was tested. Where the test for equality of slopes

failed (male thyroid/parathyroid weights; $p < 0.01$), no analysis was performed. Levene's test for equality of variances across the groups was also performed. Where this showed evidence of heterogeneity (male prostate weights; $p < 0.01$), no further analysis was performed.

Macroscopic and microscopic findings data were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.

For microscopic findings, comparisons were made between the incidence in each of the treated groups and that in the control group. Where a given finding had more than two categories, the Wilcoxon-Mann-Whitney rank sum test was performed; where only two categories were observed, Fisher's Exact test was used.

Findings:

Clinical signs and mortality:

All animals survived the duration of the study.

Thin appearance was observed in three males and one female offered 40000 ppm.

Signs noted at the daily clinical examinations, included red eyes/ears/mouth, sores/lesions and earwax. These were considered incidental as they were either infrequent or were noted at a comparable rate in the concurrent control animals. Post-dosing observations were restricted to faecal abnormalities and emesis. Soft, mucoid or liquid faeces were noted sporadically over the dosing phase in males and females offered ≥ 4000 ppm, up to eight hours following presentation of the diets. Emesis was seen in two males offered 40000 ppm in Week 1, up to eight hours following presentation of the diets.

Body weight, body weight gain, and food consumption:

There were statistically significant decreases in mean body weight or reductions in mean body weight gain for males and females at doses of 40000 ppm.

Body weight suppression from concurrent control values became statistically significant from Week 6 for males at doses offered 40000 ppm and from Week 5 for females offered 40000 ppm.

Males gained notably less weight than controls over the 13 week period, with individual growth rates for treated animals being variable, ranging from a loss of 7.5% to a gain of 11.3%. Following 13 weeks of treatment, most females at doses of 40000 ppm had similar or slightly lower body weights than initial values. However, there was an overall decrease in mean body weight principally as a result of one female exhibiting a loss of 21%.

No statistically significant alterations in mean body weight were noted for males or females offered 4000 or 12000 ppm, when compared with concurrent controls.

Food consumption in the control group, and for both sexes at doses of 4000 or 12000 ppm, was comparable and relatively stable over the treatment period (Week 1 to Week 13). When compared to concurrent controls, there was an overall reduction in mean food consumption for males and

females at doses of 40000 ppm, despite a high degree of inter-animal variability, this correlated with reductions in body weight or body weight gain.

In males offered 40000 ppm, mean consumption was similar to concurrent controls over Week 1 to Week 4. Reduced food consumption was apparent in subsequent weeks and this was statistically significant ($p < 0.001$) when compared to concurrent controls.

Consumption was consistently lower than with concurrent controls at all weekly measurement intervals for females offered 40000 ppm. Over the duration of the treatment period, the difference in consumption was statistically significant ($p < 0.001$) and marked.

Table 27: Mean body weight, body weight gain, and food consumption

	Males				**Females**			
	Dose (ppm)				**Dose (ppm)**			
	0	**4000**	**12000**	**40000**	**0**	**4000**	**12000**	**40000**
Terminal body weight (kg)	14.07	13.57	13.61	**11.16****	12.36	11.94	11.93	**9.03*****
Bodyweight gain (kg)	2.84	2.86	2.55	**0.14*****	3.01	2.38	2.47	**-0.57*****
Food consumption (g/animal/week)	2110	2111	2111	**1947*****	2100	2109	2058	**1430*****
Thin appearance	0/4	0/4	0/4	3/4	0/4	0/4	0/4	1/4

*** $p < 0.001$ different from control

** $p < 0.01$ different from control

Ophthalmoscopy:

There were no ophthalmic abnormalities noted pre-treatment or in Week 12.

Functional Observation Battery data:

Occasional statistically significant differences in mean body temperature were recorded for both sexes at doses of 12000 ppm and in males at doses of 40000 ppm, when compared to concurrent controls. In addition, in Week 3, there was a statistically significant relationship between increasing dose and response for females. These changes were transient, inconsistent and considered not to be related to treatment by the study authors.

Statistically significant differences from concurrent controls in mean heart rate were recorded sporadically for males at doses of 4000 ppm, females at doses of 12000 ppm, and there was a statistically significant relationship between increasing dose and response for females in Week 13. These changes were transient, inconsistent and considered not to be related to treatment.

There were no overt differences between control and treated animals in the incidences of observations recorded during unrestrained behaviour.

Weekly neurological examinations revealed incidences of abnormal proprioceptive paw positioning, abnormal righting postural reactions, abnormal hemihopping postural reactions, abnormal wheel barrowing postural reactions and decreased muscle tone in a few males and/or females at doses of 40000 ppm, and protrusion of the nictitating membrane in males and females

at doses ≥ 4000 ppm. In addition, there were single occurrences of abnormal hemihopping/wheel barrowing postural reactions in one female at doses of 4000 ppm in Week 9, and abnormal proprioceptive paw positioning or abnormal hemihopping postural reactions in animals offered 12000 ppm in Week 4. These recorded observations were generally infrequent, occasionally observed in control animals and are considered in some cases likely to be a result of reduced body condition.

Overall, there were no findings from any assessment indicative of any neurotoxicological effect of the test article.

Haematology and clinical chemistry:

Haematology:

Mean platelet count displayed a statistically significant increase in males at doses of 40000 ppm in Week 4 (35%), in both sexes at doses of 40000 ppm in Week 8 (47 – 53%) and in females at doses of 40000 ppm in Week 13 (69%), when compared to concurrent controls.

Sporadic differences reached statistical significance in other parameters, however, these changes were not considered treatment-related since they were slight or comparable to pre-test values.

Blood clinical chemistry:

At the mid dose and above, in males and/or females, statistically significant increases or trend toward increases in alanine aminotransferase (178-435%), alkaline phosphatase (74-249%), and γ-glutamyl transferase (67-167%) activities were observed during weeks 4, 8 and/or 13.

Statistically significant increases in aspartate aminotransferase activity (ca. 56%) and triglyceride concentration (76-82%) were observed at 40000 ppm only, during weeks 4, 8 and/or 13. Statistically significant decreases were seen mean albumin (ca. 25% males and females), A/G ratio (ca. 44% males and females), total cholesterol (52% males, 27% females) and glucose (21% males, 12% females) at 40000 ppm during weeks 4, 8 and/or 13.

There were no other blood chemistry parameters which showed a significant association with treatment.

Table 28: Haematology and clinical chemistry parameters

	Males				**Females**			
	Dose (ppm)				**Dose (ppm)**			
	0	**4000**	**12000**	**40000**	**0**	**4000**	**12000**	**40000**
Haematology (week 13)								
Mean Cell Volume (fL)	64.5	66.1	67.4	68.2	67.4	66.9	67.1	69.2
White blood cell count	9.4	9.6	9.9	**12.6***	9.1	9.6	10.2	10.6

(10^9/L)								
Neutrophils (10^9/L)	4.7	5.1	4.9	**7.3****	4.9	5.2	5.2	5.4
Monocytes (10^9/L)	0.6	0.6	0.6	**1.1***	0.6	0.5	0.5	0.6
Platelets (10^3/µL)	314	375	366	454	285	364	348	**481***
Blood clinical chemistry (week 13)								
AST (IU/L)	32	37	36	**50*****	30	31	39	**47****
ALT (IU/L)	37	30	103	**215*****	37	44	121	**198****
ALP (IU/L)	98	105	**171***	**288*****	85	114	**195*****	**297*****
γGT (IU/L)	4	3	3	**10***	3	4	5	**8***
TG (mmol/L)	0.34	0.43	0.42	**0.62****	0.38	0.39	0.42	**0.67****
ALB (g/L)	36	32	34	**27*****	37	37	35	**29*****
A/G	1.8	1.6	1.7	**1.0*****	2.4	2.0	2.0	**1.4*****
CHO (mmol/L)	6.7	5.5	5.6	**3.2*****	6.7	6.1	5.2	**4.9***
G (g/L)	20	22	20	**29****	16	18	18	22
BIL (µmol/L)	2.2	2.2	1.8	2.9	2.0	2.0	1.4	2.9
Cre (µmol/L)	62	66	64	54	57	63	**68***	61
GLU (mmol/L)	5.8	5.8	5.9	**4.6*****	5.7	5.9	5.9	**5.0***
K (mmol/L)	4.3	4.4	4.5	4.8	4.2	4.1	4.5	4.7
Ca (mmol/L)	2.80	2.72	2.75	**2.64****	2.76	2.78	2.75	2.72

* p < 0.05 different from control, ** p < 0.01 different from control, *** p < 0.001 different from control

Urine analysis:

In the highest dose group in week 12, urine volume was decreased in male animals, and specific gravity was slightly increased in both sexes (statistically significant in a dose response test).

Table 29: Urine analysis (week 12)

	Males				**Females**			
	Dose (ppm)				**Dose (ppm)**			
	0	**4000**	**12000**	**40000**	**0**	**4000**	**12000**	**40000**
Volume (ml)	339	356	255	179	175	169	136	108
Specific gravity	1.024	1.024	1.026	1.059	1.037	1.035	1.039	1.053

Sacrifice and pathology:

Microscopic findings in controls were generally infrequent, of a minor nature and consistent with the usual pattern of findings in dogs of this strain and age.

Thin appearance noted in three males and one female offered 40000 ppm generally correlated with minimal gain or body weight loss, reduced food consumption, and decreased muscle tone recorded during the functional observational battery.

Pathological findings in the liver were consistent with increases in the weight of this organ in S-2200 TG-treated males and females, and with increased aspartate aminotransferase activities, triglyceride levels seen in both sexes offered 40000 ppm, increased gamma glutamyl transferase activities in females offered 12000 ppm and both sexes offered 40000 ppm, increased alanine aminotransferase and alkaline phosphatase levels seen in both sexes offered 12000 or 40000 ppm and the decreases in total cholesterol seen in females offered 12000 ppm and in both sexes offered 40000 ppm. Therefore, the hepatic changes observed at 12000 and 40000 ppm were considered toxicologically significant. The increased globulin and total bilirubin levels seen in one male (14M) offered 40000 ppm were considered to be related to the morphological changes noted in the liver and gall bladder of this animal. On the basis that the only liver finding was centrilobular hepatocellular swelling in females and there were no associated changes in plasma enzyme levels, the changes for animals offered 4000 ppm were considered not to be toxicologically significant.

Organ weight changes in the spleen were considered not to be toxicologically significant by the notifier as there were no macroscopic or microscopic correlates, and mean values were generally within the 95% historical control reference ranges (absolute: 21.440 g to 81.380 g, relative to body weight: 0.1883% to 0.6848%; n = 103). Changes in relative kidney weight, relative brain weight and relative pituitary weight were considered not to be toxicologically significant and are probably related to the body weight loss in these animals, since the absolute weights were not statistically different, no changes were observed for related clinical chemistry parameters and there were no macro or microscopic correlates.

Decreased heart weights were considered not to be toxicologically significant, and were probably related to body weight loss in these animals, since the relative weights were not statistically significant and there were no macroscopic or microscopic correlates.

Thymus involution/atrophy was characterised by decreased cortex:medulla ratio, and in some cases an increase in tingible body macrophages. As no microscopic findings were seen at 4000 ppm and no macroscopic or microscopic findings were seen at 12000 ppm, the macroscopic change in the thymus at 4000 ppm is considered not to be related to treatment.

There was evidence of delayed sexual maturity in males (prostate, testes/epididymides) and females (ovaries). Prostate was immature in all males at 40000 ppm and pubescent in one control male, two males at doses of 4000 ppm and two males at doses of 12000 ppm. Immaturity was characterised by small, cuboidal acinar cells with no eosinophilic apical cytoplasm and little or no secretion. Pubescence was characterised by the development of a small amount of eosinophilic apical cytoplasm in some acini, with small amounts of secretion.

The testes were immature in two males at 40000 ppm. Testes were pubescent in one or two males from each group, including controls. Severe oligospermia was present in the epididymides of three males at 40000 ppm and minimal oligospermia, due to pubescence, was present in one male at doses of 12000 ppm. Immaturity was characterised by the absence of spermatids within seminiferous tubules and oligospermia in the epididymus. Pubescence was characterised by some spermatids in seminiferous tubules and epididymides. The incidence of pubescence was similar across control and treated groups.

The ovaries of two females at 40000 ppm were immature. The ovaries of all other animals in the study were pubescent. Immaturity was characterised by the absence of secondary or Graafian follicles or corpora lutea.

The changes in prostate, testes/epididymides, ovaries and thymus generally correlated with organ weights and macroscopic observations.

Decreased uterus weights in two females (29F and 32F) offered 40000 ppm were considered not to be toxicologically significant by the notifier and likely to be related to the body weight losses in these animals, since the absolute and relative weights were within background ranges (absolute: 1.622 g to 51.683 g, relative to body weight: 0.0185% to 0.4667%; n = 96) and there were no gross and/or histopathological findings observed.

Dogs used in 13 week toxicity studies are generally not fully sexually mature at sacrifice, which makes interpretation of prostate, testis and ovary findings in these studies difficult (Ronald D Hood, Developmental and Reproductive Toxicology, A practical approach. Second Edition, Taylor and Francis).

The study was started with dogs that were 20-24 weeks of age (5-6 months). At study termination, the dogs were 33-37 weeks old (about 8-9 months). Dogs achieve sexual maturity by about 7-10 months (9-10 months: Rehm 2000; 7 – 8 months: Goedkin et al 2008). Terminal sacrifice in this study therefore occurred approximately at the time dogs would be undergoing puberty, with delayed puberty at the top dose level being secondary to retarded growth and detected as immaturity.

Immaturity or delayed development of prostate, testes and ovaries can be associated with stress, weight loss and non-specific toxicity (Greaves P, Histopathology of Preclinical Toxicity Studies 2nd Edition Elsevier Science 2000, Harleman et al 1997).

‘Pubescent’ in prostate, testis and ovary and ‘immature’ in ovary were spontaneously observed in this laboratory. Organ weights of testes/epididymides, ovaries and uterus were within historical control ranges in control and treated groups; only absolute and relative prostate weights at 40000 ppm were slightly below historical control ranges (see historical control data submitted by notifier after request for clarification).

Accordingly, the immaturity of reproductive organs that was seen at 40000 ppm (a dose level which caused marked reductions in body weight, body weight gain, and food consumption) can be considered to be an indirect effect of the test article.

Table 30: Organ weights and histopathology

	Males				**Females**			
Diet concentration	**0**	**4000**	**12000**	**40000**	**0**	**4000**	**12000**	**40000**

(ppm)								
Liver and gall bladder								
Liver weight (g)	496.0	504.6	570.1	565.1	364.3	**451.5*****	**453.2*****	**387.5****
Ratio (%) to body weight	3.5	3.7	4.2	**5.1*****	2.9	**3.8*****	**3.8*****	**4.3*****
Macroscopic findings and histopathology liver								
Dark	0/4	0/4	2/4	4/4	0/4	0/4	2/4	4/4
Large	0/4	0/4	3/4	3/4	0/4	0/4	1/4	0/4
pigment	0/4	0/4	3/4	4/4	0/4	0/4	3/4	3/4
periportal/ centrilobular fibrosis	0/4	0/4	0/4	4/4	0/4	0/4	0/4	1/4
centrilobular degeneration	0/4	0/4	2/4	3/4	0/4	0/4	4/4	4/4
centrilobular hepatocellular swelling	0/4	0/4	3/4	1/4	0/4	3/4	3/4	0/4
bridging fibrosis	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4
Macroscopic and histopathology gall bladder								
Cholelithis (macrosc)	0/4	0/4	0/4	4/4	0/4	0/4	0/4	3/4
Distention	0/4	0/4	0/4	2/4	0/4	1/4	0/4	1/4
Cholelithis (histopath)	0/4	0/4	0/4	4/4	0/4	0/4	0/4	1/4
adherent bile	2/4	0/4	1/4	4/4	0/4	1/4	1/4	2/4
Kidney								
Kidney weight (g)	65.689	60.980	62.900	67.725	51.663	52.828	51.586	49.328
Ratio (%) to body weight	0.467	0.449	0.465	**0.608***	0.416	0.447	0.432	**0.552***
Brain								
Brain weight (g)	82.8	84.3	81.8	85.2	78.9	75.8	75.8	71.8
Ratio (%) to body weight	0.6	0.6	0.6	**0.8****	0.6	0.6	0.6	**0.8***
Thymus								
Thymus weight (g)	15.820	13.172	12.840	**6.914****	15.375	15.532	15.237	6.602
Ratio (%) to body weight	0.112	0.097	0.094	**0.060***	0.122	0.132	0.128	0.071
Macroscopic findings and histopathology								
Small	0/4	1/4	0/4	2/4	0/4	0/4	0/4	1/4
involution/ atrophy	0/4	0/4	0/4	2/4	0/4	0/4	0/4	2/4
agonal congestion/ haemorrhage	2/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4
Spleen								
Spleen weight (g)	47.152	44.380	46.152	69.905	46.109	44.659	53.655	40.606
Ratio (%) to body weight	0.334	0.327	0.336	**0.640***	0.367	0.381	0.456	0.463
Heart								
Heart weight (g)	108.0	100.2	107.7	**79.6****	90.2	96.3	93.9	71.4
Ratio (%) to body weight	0.8	0.7	0.8	0.7	0.7	0.8	0.8	0.8
Pituitary								

Pituitary weight (g)	0.094	0.097	0.084	0.077	0.070	0.077	0.076	0.065
Ratio (%) to body weight	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**0.001****
Prostate								
Prostate weight (g)	4.471	3.884	2.962	**1.168***	-	-	-	-
Ratio (%) to body weight	0.032	0.029	0.022	**0.011***	-	-	-	-
Macroscopic findings and histopathology								
Small	0/4	2/4	2/4	4/4	-	-	-	-
Immature	0/4	0/4	0/4	4/4	-	-	-	-
Pubescent	1/4	2/4	2/4	0/4	-	-	-	-
Mature	3/4	2/4	2/4	0/4	-	-	-	-
Testes and epididymides								
Test./epid. weight (g)	28.861	21.880	24.518	20.531	-	-	-	-
Ratio (%) to body weight	0.205	0.161	0.181	0.183	-	-	-	-
Ratio (%) to brain weight	34.9	26.0	30.0	24.1	-	-	-	-
Histopathology testes								
Immature	0/4	0/4	0/4	2/4	-	-	-	-
Pubescent	2/4	1/4	1/4	1/4	-	-	-	-
Mature	2/4	3/4	3/4	1/4	-	-	-	-
Segmental hypoplasia	1/4	3/4	0/4	1/4	-	-	-	-
Histopathology epididymides								
Oligospermia/ spermatocytes	0/4	0/4	1/4	3/4	-	-	-	-
Ovaries								
Ovaries weight (g)	-	-	-	-	0.758	0.999	0.833	0.744
Ratio (%) to body weight	-	-	-	-	0.006	0.008	0.007	0.008
Histopathology								
Immature	-	-	-	-	0/4	0/4	0/4	2/4
Pubescent	-	-	-	-	4/4	4/4	4/4	2/4
Uterus								
Uterus weight (g)	-	-	-	-	4.198	5.623	5.631	2.702
Ratio (%) to body weight	-	-	-	-	0.034	0.047	0.048	0.030

* $p < 0.05$ different from control; ** $p < 0.01$ different from control; *** $p < 0.001$ different from control.

Table 31: Historical control data submitted: Jan 1998-Mar 2007, 27-52 weeks of age

	Males				Females			
	Mean	**n**	**95% ref range low**	**95% ref range high**	**Mean**	**n**	**95% ref range low**	**95% ref range high**
Terminal Body Weight (g)	11543	148	7013	15603	10172	147	6865	13719
Testes/Epididymides (g)	21.279	140	5.313	35.359	-	-	-	-
Testes/Epididymides ratio (%) to bw	0.1807	140	0.0731	0.2643	-	-	-	-
Testes/Epididymides ratio (%) to brain weight	25.97	140	7.20	43.51	-	-	-	-
Ovaries (g)	-	-	-	-	1.171	146	0.525	3.630
Ovaries ratio (%) to bw	-	-	-	-	0.0114	146	0.0058	0.0345
Thymus (g)	14.908	72	5.331	26.766	13.850	72	6.270	28.299
Thymus ratio (%) to bw	0.1320	72	0.0576	0.2458	0.1398	72	0.0560	0.2692
Prostate (g)	5.589	144	1.289	13.730	-	-	-	-
Prostate ratio (%) to bw	0.0472	144	0.0113	0.1093	-	-	-	-
Uterus (g)	-	-	-	-	n.a.	96	1.622	51.683

n.a. not available

Conclusion:

In the 90 days study in dog the NOAEL for males and females is proposed at 4000 ppm (90.9 mg/kg bw/day for male and 102.7 mg/kg bw/day for female animals, based on increased liver weight, histopathological changes in the liver, and increased alkaline phosphatase levels. Liver weight increases in females dosed at 4000 ppm (> 20%) were not observed in the 52 week toxicity study in dogs and were therefore not considered for the derivation of the NOAEL of the 90 day study.

1 year feeding study

Reference:	S-2200 Technical Grade: 52 Week Oral (Dietary) Administration Toxicity Study in the Dog
Author(s), year:	Beck, W.; 2012a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0071
Guideline(s):	OECD 452, US EPA OPPTS 870.4100, JMAFF 2-1-14, EC No 440/2008 B30
GLP:	Yes (Laboratory certified by national authority)
Deviations:	Minor deviations not affecting the validity of the study
Validity:	Yes

Material and methods:

Test Material: S-2200 Technical Grade (S-2200 TG)
Lot/Batch: ST-0811G
Purity: 93.4%
Stability of test compound: Expiry date stated as 21 November 2011 (after end of treatment)
Vehicle: none

Test animals:
Species: Dog
Strain: Beagle
Age: 27 – 34 weeks at beginning of dosing
Weight at dosing: Males: 10.02 – 13.42 kg; Females: 8.86 – 12.20 kg
Source: Harlan, Bicester, UK
Diet: 5L66 Certified High Density Canine Diet (IPS Product Supplies Ltd, London, UK); 300 g per day

Animal assignment and treatment:

Twenty healthy animals per sex were selected based on the results of pre-test examinations. Animals were randomly distributed into groups of 4 animals/sex/group based on the most recent body weight data, except for separation of familial animals.

The test article was administered orally by incorporation into the diet. Each animal had ad libitum access to the dietary formulations for approximately 8 hours/day for at least 52 weeks. Control animals received untreated diet.

Diet preparation, analysis and administration:

Fresh batches of dietary formulation were prepared weekly. All diets were hand mixed with mains water into clumps of irregular size and shape. The formulations were stored at room temperature in the dark before administration.

Accuracy and homogeneity of preparation was confirmed by analysis. Stability in diet for at least 9 days at ambient temperature was established before the study started.

Although there were some deviations to the protocol with regards to the analysis of the test formulations, the data was sufficient to confirm homogeneity and stability, and there is considered to be no impact to the study integrity.

Table 32: Diet concentration and daily dose levels

	Males					**Females**				
Diet concentration (ppm)	**0**	**200**	**800**	**4000**	**8000**	**0**	**200**	**800**	**4000**	**8000**
mg test material/kg bw/day	0	4.3	19.2	92.0	180.7	0	4.5	20.4	92.0	225.7

Clinical observations:

All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a daily detailed physical examination. Animals were observed approximately 1 and 4 hours after presentation of the diet. A number of animals from both the control and treatment groups received veterinary treatments throughout the study period. The need for veterinary treatment was not dose related.

All animals were subjected to a battery of behavioural tests and observations before initiation of treatment and at once weekly intervals thereafter. The unrestrained behaviour of each animal was observed in an examination room for approximately 1 to 2 minutes.

Observations included the following:

Level of consciousness	General level of activity
Head posture	Head co-ordination
Neck posture	Circling
Limb posture (standing)	Gait
Tremors	Unusual behavior

Visual fixating response test and auditory startle tests were assessed at the end of the observation period.

Following the unrestrained observations all animals were subjected to neurological examinations each week.

Examinations included the following:

Proprioceptive paw positioning	Righting postural reaction
Hemihopping postural reactions	Wheel barrowing postural reactions
Muscle tone	Palpebral closure
Nictitating membrane	Palpebral reflex
Eye position	Eye movement
Pupil size	Pupillary light reflex
Lacrimation	Salivation
General reactions	General clinical observations

Body weight, general clinical signs, heart rate and rectal temperatures were taken as part of the data set for assessment of neurobehavioral effects of test articles.

Food consumption and body weight:

Food consumption for all animals was measured daily from the time of allocation until termination of the dosing period.

Weekly compound consumption was calculated as mg/kg bw/day and also as a total consumption over the entire treatment period (mg/kg bw/day).

Body weight was measured in all animals once weekly throughout the dosing period, from Week -3 until termination.

Ophthalmoscopic observation:

A mydriatic agent was instilled into the eyes before examinations. Eyes of all animals were observed once pre-test and at Week 51.

Haematology and clinical chemistry:

Blood was sampled from all animals before the start of treatment (Week -1) and at weeks 13, 26, and 52 of the administration period (sampling was performed after animals were fasted overnight). Samples were taken from jugular vein and collected into EDTA (for cell parameters), trisodium citrate (for clotting function), and lithium heparin (blood chemistry).

1.0 mL (nominal) of blood was collected from each animal for haematology (0.5 mL for coagulation tests and 0.5 mL for other tests). The blood sample collected into trisodium citrate anticoagulant from one female in Week 52 was unsuitable for analysis, so blood samples were recollected and re-examined on week 53.

The following parameters were examined:

Haematology: Haemoglobin concentration (HGB), Red blood cell count (RBC), Mean cell haemoglobin (MCH), Mean cell haemoglobin concentration (MCHC), Mean cell volume (MCV), Packed cell volume (PCV), Platelet count (PLT), Total and differential white blood cell count, Reticulocytes, Prothrombin time (PT), and Activated partial thromboplastin time (APTT)

Blood chemistry: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), γ-Glutamyl transferase (GGT), Sodium (Na), Potassium (K), Calcium (Ca), Inorganic phosphorus (IP), Chloride (Cl), Triglycerides (TG), Total protein (TP), Albumin (Alb), Globulin (G), Albumin-globulin ratio (A/G), Total cholesterol (Cho), Glucose (Glu), Urea (U), Total bilirubin (Bil), and Creatinine (Cre).

Urine analysis:

Urine samples were collected overnight from all animals before the start of treatment (Week -1) and in Weeks 12, 25, and 51 of the administration period; food and water were removed during collection. In Week 25, two of the male urine samples showed a blue discolouration. Following a thorough investigation, this was considered due to trace contamination of the apparatus with a dyestuff and not considered to impact the study integrity. Fresh samples were obtained overnight from all males in Week 25. Additionally, in Week 51, two further male urine samples showed a blue discolouration. The cages were visually inspected prior to collection and considered to be clean. Prior to the female collection, all cages were thoroughly power washed after being left to soak in detergent. Subsequently, the discoloured male samples were repeated in Week 52.

Parameters examined: Urinary volume (Vol), Colour (Col), Turbidity, Specific Gravity (S.G.), pH, Protein (Pro), Glucose (Glu), Ketones (Ket), Urobilinogen (Uro), Bilirubin (Bil), Blood, and Microscopic sediment.

Sacrifice and pathology:

All animals survived until scheduled sacrifice, and were sacrificed with sodium thiopentone, and exsanguinated from the major blood vessels.

The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes (with epididymides), thymus, thyroids (with parathyroids), and uterus (including cervix). Bilateral organs were weighed together.

Histopathological examination: After necropsy, the following organs and tissues collected from all animals were fixed in 10% neutral buffered formalin, except bone marrow smears which were fixed in methanol, testes and epididymides which were fixed in Bouin's solution and eyes/optic nerves fixed in Davidson's fluid.

Adrenals	Ovaries
Aorta	Oviducts
Bone and marrow (sternum)	Pancreas
Brain	Pituitary
Caecum	Prostate
Colon	Rectum
Duodenum	Salivary gland – mandibular
Eyes and Optic nerves	Salivary gland – Sublingual, parotid
Bone with marrow (femur) & articular surface	Sciatic nerves
Gallbladder	Skin and subcutaneous tissue
Gross lesions	Spinal cord (cervical, thoracic, lumbar)
Heart	Spleen
Ileum	Sternum with bone marrow
Jejunum	Stifle joint
Kidneys	Stomach
Larynx	Testes and epididymides
Liver	Thymus
Lungs with bronchi and bronchioles	Thyroids and parathyroids
Mammary glands	Tongue
Mandibular lymph nodes	Trachea
Mesenteric lymph nodes	Ureters
Muscle	Urinary bladder
Nasal cavity	Uterus (including cervix)
Nasopharynx	Vagina
Oesophagus	

Statistical analysis:

Necropsy body weights, organ weights and organ weight to body weight ratios were analysed using one-way analysis of variance, separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Absolute body weights, body weight gains, food consumption, body temperatures, heart rates, haematology, blood biochemistry and urine analysis variables were analysed using two-way ANOVA. Levene's test for equality of variances across groups, between sexes and for any interaction was performed. Where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex separately, a linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of a sex effect only ($p < 0.01$ for both untransformed and log-transformed data), the data were analysed using one-way ANOVA for each sex separately, as previously described.

Where Levene's test showed evidence of group effects, sex effects or a sex by group interaction ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Clinical pathology parameters with values above or below the limit of the assay were analysed using non-parametric methods, as described above. Where such values occurred in one sex only, data for the other sex were analysed using one-way ANOVA.

All macroscopic and microscopic findings data, for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk.

For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher’s Exact test.

For microscopic findings, comparisons were made between the incidence in each of the treated groups and that in the control group. Where a given finding had more than two categories, the Wilcoxon-Mann-Whitney rank sum test was performed; where only two categories were observed, Fisher’s Exact test was used.

Findings:

Clinical signs and mortality:

All animals survived the duration of the study.

Thin appearance was observed in one female offered 8000 ppm, and this was considered to be correlated with body weight loss and occasional incidences of decreased muscle tone recorded during the functional observation battery. Other clinical observations noted at the daily

examinations in treated animals were considered incidental as they were infrequent, associated with the female sexual cycle or typical findings of dogs held under laboratory conditions.

Body weight, body weight gain, and food consumption:

No statistically significant alterations in mean body weights or mean body weight gains were noted in treated animals, when compared to concurrent controls.

Following 52 weeks of treatment, females offered 8000 ppm displayed a body weight gain lower than concurrent controls. This reduced growth rate was due to one female animal exhibiting an overall bodyweight loss of 6.0%.

Table 33: Mean body weight, body weight gain, and food consumption

	Males					Females				
	Dose (ppm)					Dose (ppm)				
	0	**200**	**800**	**4000**	**8000**	**0**	**200**	**800**	**4000**	**8000**
Terminal body weight (kg)	14,64	15,22	13,29	14,10	13,70	12,92	12,71	12,63	13,36	11,05
Bodyweight gain (kg)	2,97	3,88	1,82	2,58	2,66	2,68	2,36	2,57	3,19	0,81
Food consumption (g/animal/week)	2107	2110	2112	2110	2111	2067	1920	2089	1993	2094

Ophthalmoscopy:

There were no ophthalmic abnormalities noted pre-treatment or in Week 52.

Functional observation battery data:

Overall, there were no findings from any assessment indicative of any neurotoxicological effect of the test article.

Occasional statistically significant differences in mean body temperature were recorded for males offered ≥ 800 ppm and females offered ≥ 200 ppm, when compared to concurrent controls. In addition, there was a statistically significant relationship between increasing dose and response for males in Weeks 15, 18, 28, 34 and 43 and for females in Weeks 7, 8, 9, 11 and 15. These changes were transient, inconsistent and considered not to be related to treatment.

Statistically significant differences from concurrent controls in mean heart rate were recorded sporadically for males offered ≥ 200 ppm and females offered 800 or 4000 ppm. These changes were transient, inconsistent and considered not to be related to treatment.

There were no overt differences between concurrent control and treated animals in the incidences of observations recorded for unrestrained behaviour.

Neurological examinations showed one or more incidences of abnormal proprioceptive paw positioning, abnormal righting postural reactions, abnormal hemihopping postural reactions, abnormal wheelbarrowing postural reactions, abnormal muscle tone, abnormal palpebral closure, protrusion of the nictitating membrane, strabismus/protrusion of the palpebral reflex , bilateral constriction/dilation of pupil size, abnormal pupillary light reflex, increased lacrimation,

increased salivation and untypical general reactions. These observations were infrequent and occasionally observed in concurrent control animals or before treatment was initiated.

Haematology and clinical chemistry:

For clinical biochemistry, several statistically significant differences between controls and treated groups were observed.

Statistically increased aspartate aminotransferase activity in Week 26 for males offered 8000 ppm is considered not to be treatment-related as this finding is largely reflective of an increase for one male with values for the other animals in this group being within historical control range. Furthermore, the plasma activity for the same male in Week 52 was similar to that determined before treatment. The changes at Week 52 for another male offered 8000 ppm are considered to be treatment related.

Disturbances to alanine aminotransferase, alkaline phosphatase (800 ppm only), gamma glutamyltransferase and/or total cholesterol for animals offered 200 or 800 ppm were considered not to be toxicologically significant as they were small in magnitude, inconsistent over time and between sexes and/or lacked a histopathological correlate. However, the increased alkaline phosphatase activities for males offered 4000 ppm at Week 52, and males and females offered 8000 ppm at Weeks 13, 26 and 52, are considered to be treatment-related.

The statistically higher triglyceride level observed at Week 26 in males offered 4000 ppm was considered not to be toxicologically significant since the mean value is within the historical control range. The increased triglyceride level for one male offered 4000 ppm and two males offered 8000 ppm at Week 13 are considered not to be toxicologically significant since the values for these animals at Week 52 were similar to that determined before treatment was initiated. The changes for one male offered 4000 ppm at Week 13 and Week 52, and one male offered 8000 ppm at Week 52, are considered to be treatment related.

Albumin levels were statistically significantly decreased in females offered 8000 ppm at Weeks 26 and 52, when compared to concurrent controls.

Statistically decreased total protein and calcium levels in females offered 8000 ppm at Weeks 13 and 26 and at Weeks13, 26 and 52, respectively, are considered not to be treatment-related as it may be in part reflective of decreased albumin concentrations (Meuten DJ, Relationship of serum total calcium to albumin and total protein in dogs. J Am Vet Med Assoc. 1982;180(1):63-7). Additionally, all individual values were within the historical control ranges and/or differences were similar to those recorded pre-treatment.

Total cholesterol was statistically decreased at Week 26 in females offered 200, 800 or 8000 ppm and at Week 52 in females offered 800 or 8000 ppm, when compared to concurrent controls. As the mean values were similar to those determined before treatment was initiated, the differences at Week 26 are considered not to be toxicologically important.

Occasional differences reached statistical significance in other parameters, however, these changes were not considered treatment-related since they were slight, within the historical control range, comparable to pre-test values or there were no significant differences between sexes and collection points.

Table 34: Haematology and clinical chemistry parameters (weeks 13, 26, and 51)

	Males					Females				
	Dose (ppm)					Dose (ppm)				
	0	**200**	**800**	**4000**	**8000**	**0**	**200**	**800**	**4000**	**8000**
AST (IU/L)	32	38	29	34	42	28	28	26	32	36
	30	30	29	29	**61***	26	30	28	23	28
	29	30	29	27	37	26	24	29	28	30
ALT (IU/L)	38	42	44	46	136	29	36	38	39	79
	42	48	48	43	113	31	36	46	31	57
	40	53	51	47	128	29	35	50	33	59
ALP (IU/L)	63	68	103	120	**178***	91	76	70	91	**207***
	53	52	100	120	**140***	75	79	66	78	**198****
	41	43	73	**112****	**147****	55	64	53	69	**185****
γGT (IU/L)	2	2	3	3	4	2	3	3	2	3
	3	5	3	5	3	2	2	4	3	4
	3	3	4	4	4	3	3	3	4	5
TP (g/L)	55	55	56	57	57	60	58	57	56	**55****
	58	58	59	59	58	62	62	60	61	**57***
	58	59	56	59	59	61	61	59	59	57
Alb (g/L)	35	36	36	37	36	39	37	38	37	35
	36	35	34	35	33	39	38	36	37	**34***
	35	35	33	37	33	39	37	37	36	**34****
A/G ratio	1.8	1.9	1.8	1.9	1.6	1.9	1.9	1.9	2.1	1.8
	1.6	1.6	1.4	1.4	1.4	1.6	1.6	1.5	1.6	1.5
	1.5	1.5	1.5	1.7	1.3	1.8	1.6	1.6	1.6	1.5
Cho (mmol/L)	7.1	5.9	6.6	6.9	6.1	8.3	7.0	6.1	5.9	6.4
	6.7	5.7	6.7	6.7	5.8	8.2	**6.3***	**5.9****	6.6	**6.0***
	6.0	5.1	5.7	6.3	5.5	7.5	6.1	**5.5***	5.9	**5.4***
TG (mmol/L)	0.37	0.28	0.45	0.53	0.48	0.59	0.63	0.50	0.49	0.51
	0.33	0.30	0.44	**0.49***	0.45	0.51	0.48	0.48	0.52	0.50
	0.40	0.36	0.42	0.53	0.52	0.55	0.60	0.60	0.58	0.52
Ca (mmol/L)	2.83	2.79	2.76	2.81	2.80	2.89	2.88	2.86	2.84	**2.70***
	2.66	2.67	2.60	2.66	2.61	2.74	2.76	2.72	2.74	**2.59***
	2.63	2.63	2.57	2.69	2.57	2.71	2.70	2.66	2.66	**2.53***

* $p < 0.05$ different from control, ** $p < 0.01$ different from control

Urine analysis:

There were no treatment-related effects on urinary parameters measured at the Week 12, 25 or 51.

The mean volume of urine voided by females offered 200 or 800 ppm at Week 12 was statistically decreased when compared to concurrent controls. However, as the volumes were similar to those measured before treatment, no dose response was observed and no statistical

differences from concurrent controls were observed in males at Week 12 or in both sexes at Weeks 25 or 51, changes are considered to be incidental and not related to treatment.

Sacrifice and pathology:

Organ Weights:

Increased mean liver weights were found in males offered ≥ 4000 ppm and females offered 8000 ppm, with a statistically significant increase in the relative liver-to-bodyweight for females offered 8000 ppm compared to controls.

Decreases in absolute kidney weight were considered not to be toxicologically significant since the relative weights were not statistically different, all individual values were within historical control limits, no changes were observed for related clinical chemistry parameters and there were no macroscopic or microscopic correlates.

Organ weight changes in the adrenals and testes/epididymides were considered not to be toxicologically significant as they were either inconsistent between sexes, there were no macroscopic or microscopic correlates, values were within historical control limits or there was a lack of a clear dose-response relationship.

Gross and Histopathology:

Most tissues were macroscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in animals of this strain and age.

Dark liver was observed for one male and three females offered 8000 ppm, and correlated with findings recorded microscopically.

There were no other macroscopic findings suggestive of treatment-related effects.

Microscopic findings were generally infrequent, of a minor nature and consistent with the usual pattern of findings in animals of this strain and age.

In the liver, hepatocyte hypertrophy was recorded for males and females offered 4000 or 8000 ppm. Increased levels of hepatocyte pigment were recorded for males offered 4000 or 8000 ppm and females offered 8000 ppm, when compared with controls. The incidence of hepatocyte hypertrophy and hepatocyte pigment achieved statistical significance ($p < 0.05$) in males and females offered 8000 ppm, when compared with the controls. There was also a marginal increase in pigmented macrophages in males and females offered 8000 ppm, when compared with the controls. Additionally, centrilobular degeneration was recorded for one male and one female offered 8000 ppm, and portal fibrosis/bile duct proliferation was recorded for one male offered 8000 ppm.

Agonal congestion/haemorrhage was recorded for one male and three females offered 8000 ppm, this finding correlated with the observed dark liver that was recorded during the macroscopic examination.

Hepatocyte hypertrophy was characterised by hepatocytes in the centrilobular/mid-zonal area with increased pale eosinophilic staining cytoplasm. Hepatocyte pigment was characterised by the presence of small golden brown cytoplasmic granules, primarily in hepatocytes in the centrilobular and periportal zones. Pigmented macrophages were characterised by macrophages

in the portal area and in the hepatic sinusoids with dark brown cytoplasmic pigment. The hepatocyte pigment and macrophage pigment was identified as lipofuscin using Schmorl's and Long Ziehl Neelsen stains. Centrilobular degeneration was characterised by the presence of degenerating hepatocytes in the centrilobular zone, with occasional single cell necrosis. Portal fibrosis/bile duct proliferation was characterised by the presence of proliferating bile duct cells and fibroblasts, with an overall increase in fibrous tissue in the portal tracts. The increase in lipofuscin on this study was considered to be related to hepatocyte hypertrophy and/or cellular degeneration.

The liver of animals offered 200 or 800 ppm was comparable to the controls.

There were no other microscopic findings suggestive of treatment-related effects.

Table 35: Organ weights and histopathology

	Males					**Females**				
Diet concentration (ppm)	**0**	**200**	**800**	**4000**	**8000**	**0**	**200**	**800**	**4000**	**8000**
Liver										
Liver weight (g)	437	425	409	484	488	429	437	437	448	455
Ratio (%) to body weight	3.0	2.8	3.1	3.4	3.6	3.3	3.4	3.5	3.4	**4.2***
Macroscopic and histopathology liver										
Dark	0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	3/4
hepatocyte hypertrophy	0	0	0	2	3	0	0	0	1	4
hepatocyte pigment	1	0	1	3	4	1	1	0	1	4
pigmented macrophages	1	0	0	0	2	1	0	0	1	2
portal fibrosis/bile duct proliferation	0	0	0	0	1	0	0	0	0	0
centrilobular degeneration	0	0	0	0	1	0	0	0	0	1
agonal congestion/haemorrhage	0	0	0	0	1	0	0	0	0	3
Kidney										
Kidney (g)	73.9	65.0	61.7	**60.6***	**60.0***	57.3	58.5	58.4	53.1	54.9
Ratio (%) to body weight	0.508	0.428	0.469	0.431	0.437	0.442	0.460	0.467	0.400	0.509
Adrenals										
Ratio (%) to body weight	0.010	0.010	0.010	0.010	0.011	0.013	0.015	0.017	**0.009***	**0.017***
Testes/ epididymides										
Testes/epididymides (g)	32.3	25.2	**24.3***	27.5	25.3	-	-	-	-	-
Ratio (%) to body weight	0.220	**0.166***	0.186	0.196	0.184	-	-	-	-	-

* $p < 0.05$ different from control; ** $p < 0.01$ different from control; *** $p < 0.001$ different from control.

Conclusion:

Mandestrobin administration of 8000 ppm was associated with increased absolute and relative liver weights, hepatocyte hypertrophy and hepatocyte pigment and disturbances to clinical biochemistry parameters (increased alkaline phosphatase, γ-glutamyltransferase and triglycerides) in both genders. At 4000 ppm, male animals showed increased ALP-activity and hepatocyte hypertrophy and pigmentation. The livers of female animals at this dose level were comparable to controls.

Based on these indicators of changes in the liver, the No Observable Adverse Effect Level (NOAEL) for males was considered to be 800 ppm (19.2 mg/kg bw/day), and the NOAEL for females was concluded to be 4000 ppm (92.0 mg/kg bw/day).

4.7.1.2 Repeated dose toxicity: inhalation

No repeated dose inhalation studies are available.

4.7.1.3 Repeated dose toxicity: dermal

28 days dermal toxicity study in rats

Reference:	A 28-Day Repeated Dose Dermal Toxicity Study of S-2200 Technical Grade in Rats
Author(s), year:	Ogata, H.; 2011
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0022
Guideline(s):	OECD 410, EC Guideline B.9 OJEC No L 383 A/144, EPA OPPTS 870.3200, and Japanese MAFF 12 Nousan 8147 (2000)
GLP:	Yes (lab certified by National Authority)
Deviations:	No
Validity:	Yes

Material and methods:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	The stability of the test compound during the study period was confirmed
Vehicle:	Water for injection
Test animals:	
Species:	Rat
Strain:	Slc: Wistar
Age:	8 weeks at start of treatment
Weight at dosing:	Males: 123.2 – 146.8 g; Females: 97.5 – 125.6 g (upon receipt)
Source:	Japan SLC, Inc., Shizuoka, 431-1103, Japan
Diet:	CRF-1 pellet diet (Oriental Yeast Co., Ltd) *ad libitum*

Animal assignment and treatment:

10 animals per sex were assigned to each group by the stratified sequenced randomization method on the basis of body weight of the day, and it was confirmed that the weight variation of animals used was within ± 20% of the mean weight for each sex.

About 24 hours before the first administration, hair on the administration sites (dorsal region, approximately 5 cm × 6 cm) of animals was clipped off with an electric clipper (Speedik DC-6, Blade: 0.1 mm, Shimizu E.C.) while taking care not to damage the skin. After that, hair clipping was performed for all animals on days 7, 14, 21, and 28.

Dose levels were 100, 300 and 1000 mg/kg bw/day, applied to the skin for 6 hours/day.

The required amount of test compound was weighed on a piece of medical paper based on the latest body weights.

The administration area was equivalent to 10% of the total body surface (approximately 4 cm × 5 cm). The required amount of dosing formulation (test compound) was uniformly transferred on a gauze sheet (approximately 4 cm × 5 cm, 1 piece, Yamato Kojo Co., Ltd.) lined with an impermeable sheet (BlendermTM, 3M Health Care), and the gauze sheet was moistened sufficiently with about 0.3 mL vehicle (water for injection). The administration site was covered with the gauze sheet and occluded with an elastic bandage (SILKYTEX, ALCARE Co., Ltd.) for at least 6 hours. The skin from the axillary to the chest region was covered with a gauze sheet to protect it from the bandage.

After about 6-hour occlusive application, the test compound, gauze sheet, and elastic bandage were removed.

The administration area was wiped once with a sheet of gauze (approximately 4 cm × 5 cm, 1 piece) immersed in lukewarm water (this operation was repeated 3 times), and then wiped with dry tissue paper to remove fluid on the skin. The animals were restrained with neck collars during the administration period, except during the body weight measurement, ophthalmology, and pooled urine sampling. The animals in the control group were treated with vehicle only, and were handled in the identical manner to the test group subjects.

Clinical observations:

Clinical signs and mortality were observed twice daily, before administration and after the end of administration (after removal of dosing formulation).

Detailed clinical observation was conducted before the start of administration and in weeks 1, 2, 3, and 4 of administration (after removal of dosing formulation). Animals were observed in an open field for 2 minutes. The observation results were evaluated according to scoring criteria.

Cageside observations included posture, convulsions, stereotypies and bizarre behaviour, and tremors. Hand held observations included reactivity to handling, vocalizations, tremors, twitches, convulsions, respiration, salivation, lacrimation, pupil size, exophthalmos, ocular or nasal secretions, skin, piloerection, soiled fur, skin colour, incontinence of urine, muscle tone, and body temperature. Open field observations included arousal, gait, stereotypies and bizarre behaviour, ptosis, diarrhoea, defecation, and urination.

Food consumption and body weight:

Body weights were measured for all animals on days 1, 8, 15, 22, and 28, and at necropsy. Food consumption was measured for all animals on days 1, 8, 15, 22, and 28.

Ophthalmoscopic observation:

Ophthalmoscopy was conducted before the start of administration and in week 4 before dosing. Light reflexes were confirmed using a direct ophthalmoscope (HEINE alpha+ Ophthalmoscope). A slit lamp (SL-15, Kowa) was used with mydriatic solution to inspect the cornea, iris, lens and vitreous body, and a binocular indirect ophthalmoscope (OMEGA2000, Heine Optotechnik GmbH & Co. KG) was used to inspect the fundus oculi.

Haematology and clinical chemistry:

The animals were examined at necropsy after the end of the administration period. All animals were anesthetized with intraperitoneal injection of sodium pentobarbital and blood was collected from posterior vena cava.

For examination of the coagulation system, 0.9 mL of blood was collected into a glass tube containing trisodium citrate. For the examination of other items, remaining blood was collected into a container containing an anticoagulant (EDTA-2K). The animals were fasted for 16 to 22 hours before blood sampling. Parameters measured were:

Haematology:

Leukocytes, Differential leukocyte count: (Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils) Erythrocytes (RBC), Haemoglobin concentration (Hgb), Haematocrit, Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Reticulocytes, Platelets, Prothrombin time (PT), Activated partial thromboplastin time (APTT)

Blood biochemistry:

Total protein, Albumin, Albumin/globulin ratio (A/G ratio), Total bilirubin (T. bilirubin), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), γ-Glutamyl transpeptidase (γ-GTP), Alkaline phosphatase (ALP), Total cholesterol (T. Cholesterol), Triglycerides, Phospholipids, Glucose, Blood urea nitrogen (BUN), Creatinine, Inorganic phosphorus (IP), Calcium (Ca), Sodium (Na), Potassium (K), Chloride (Cl).

Urinalysis:

Urinalysis was conducted in week 4.

Fresh urine samples were collected in the morning before dosing, using metabolic cages, and urine samples were collected successively for about 24 hours (pooled urine). The following parameters were measured: Volume, Gravity, and Colour were all examined using 24 hour pooled samples. pH, protein, glucose, ketone body, occult blood, and urobilinogen were examined using fresh urine samples with Pretest 8aII (Wako Pure Chemical Industries Ltd.). Urine sediments were obtained by centrifugation and examined for epithelial cells, erythrocytes, leukocytes, casts, and crystals (phosphates and oxalates).

Sacrifice and pathology:

At the end of the administration period, animals were euthanized by exsanguination after blood sampling. All organs and tissues were immediately examined macroscopically.

After necropsy, organs were weighed (absolute weight) and the ratio of organ weight to body weight (relative weight) was calculated on the basis of body weight measured on the day of necropsy. Paired organs were measured separately and the total weight was also calculated.

Organs and tissues were fixed in 10% neutral buffered formalin (the eyes, optic nerves and harderian gland were pre-fixed in Davidson's solution, and the testes and epididymides were pre-fixed in Bouin's solution). In all animals in control and high dose groups, embedded block specimens (submaxillary lymph node: left side, parathyroid gland: left side (Nos.376 and 377: right side), other bilateral organs/tissues: left side) were prepared, sectioned with paraffin and stained with haematoxylin and eosin (HE) for microscopic examination. In addition, all gross lesions were examined microscopically in the same manner.

Since examination of the high dose group showed no changes related to the test compound administration, the histopathological examination of the middle and low dose groups, except for the examination of gross lesions, was not conducted.

Sampling and examination of organs were performed as follows:

Tongue, Larynx/Pharynx, Oesophagus, Stomach, Duodenum, Jejunum, Ileum (with Peyer's patch), Caecum, Colon, Rectum, Submaxillary grand (with sublingual gland), Parotid gland, Liver, Pancreas, Trachea, Lung (with bronchi), Thymus, Submaxillary lymph node, Mesenteric lymph node, Spleen, Heart, Aorta, Kidney, Urinary bladder, Prostate, Seminal vesicle, Testis, Epididymis, Ovary, Uterus, Vagina, Mammary gland, Pituitary, Thyroid (with parathyroid) *[1], Adrenal, Brain *[2], Spinal cord (cervical to lumbar) *[3], Optic nerve, Sciatic nerve, Eye, Harderian gland, M. biceps femoris, Sternum (with bone marrow), Femur (with bone marrow), Integument (lower abdominal), Nasal cavity, Treated site, Other gross lesions.

*[1]: The parathyroid was examined on either side
*[2]: Cerebrum, cerebellum and medulla/pons
*[3]: Cervical, thoracic and lumbar region

Findings:

Clinical signs and mortality:
All animals survived until the scheduled sacrifice. No treatment-related clinical signs were detected.

Body weight and body weight gain:
There were no differences in bodyweight and bodyweight gain between treated groups and control animals during and at the end of the study.

Food consumption:
Food consumption was unaffected by treatment.

Ophthalmoscopy:

No abnormalities were seen in any treated group.

Haematology and clinical chemistry:
No changes related to the administration of the test compound were seen in any group.

Sacrifice and pathology:
No changes related to the administration of the test compound were seen in any group.

Conclusion:

There was no toxicological change related to administration of the test compound in any parameter. Therefore, the NOAEL of mandestrobin under the current study conditions is concluded to be 1000 mg/kg bw/day (the highest dose tested) for both males and females.

4.7.1.4 Repeated dose toxicity: other routes

No data on other routes available.

4.7.1.5 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.7.1.6 Other relevant information

Several *in vivo* and *in vitro* mechanistic studies were performed, and two detailed position papers were submitted dealing with (i) liver and thyroid effects and (ii) ovary issues. These studies and position papers are summarised in section *4.12.1.3 Specific investigations: other studies*.

4.7.1.7 Summary and discussion of repeated dose toxicity

A series of studies was carried out to investigate the effects of orally administered mandestrobin in rats (one 90-day study), mice (one 90-day study) and dogs (one 90-day and one 1-year study) following repeated exposure over subchronic periods. In addition, effects after repeated exposure via the dermal route were also investigated in the rat (one 28-day study). Furthermore, the effects of mandestrobin via the oral route over a chronic period were investigated in a 2-year study in rats and in a 78-week study in mice.

In the 90-days rat study, the NOAEL for females was set at 4000 ppm (320.1 mg/kg bw/day) based on liver weight increase of close to 20%, hepatocellular hypertrophy, increased cholesterol, and thyroid follicular cell hypertrophy at 10000 ppm. The NOAEL for male rats was set at 800 ppm (54 mg/kg bw/day) based on absolute (13.6%) and relative (15.5%) liver weight increase and hepatocellular hypertrophy.
The mechanistic basis for increases in liver weight, hepatocellular hypertrophy, and follicular cell hypertrophy in the thyroid gland observed from 4000 ppm is considered to be liver enzyme

induction and increased catabolism of thyroid hormones in a manner similar to the observed effects induced by phenobarbital (see also position paper Yamada 2012a and study Asano 2012e). These effects are not considered relevant for human risk assessment.

In a 90-days toxicity study in the mouse (0, 1750, 3500 and 7000 ppm mandestrobin), liver weight increases were noted in all treated groups in both sexes. Because an adaptive mechanism is ascribable for the increase in liver weights rather than a pathological effect (absence of any biochemical or histological markers of liver pathology), the increases were considered non-adverse.
In the absence of other treatment-related adverse findings, the NOAEL is considered to be 7000 ppm (807.3 mg/kg bw/day for males and 1111.2 mg/kg bw/day for females, respectively).

In the 90-days study in dogs the NOAEL is proposed at 4000 ppm (90.9 mg/kg bw/day for male and 102.7 mg/kg bw/day for female animals), based on increased liver weight, histopathological changes in the liver (pigmentation and centrilobular degeneration), and increased alkaline phosphatase levels.

In the 1-year dog study the NOAEL for males is proposed at 800 ppm (19.2 mg/kg bw/day), and the NOAEL for females at 4000 ppm (92.0 mg/kg bw/day). Mandestrobin administration of 8000 ppm was associated with increased absolute and relative liver weights, hepatocyte hypertrophy, hepatocyte pigment and disturbances to clinical biochemistry parameters (increased alkaline phosphatase, γ-glutamyltransferase and triglycerides) in both genders. At 4000 ppm, male animals showed increased ALP-activity, hepatocyte hypertrophy, and pigmentation. The livers of female animals showed similar changes at the next higher dose level (8000 ppm).

In the 28-days dermal toxicity study in rats, no toxicological change related to administration of mandestrobin was observed in any tested parameter. Therefore, the NOAEL is concluded to be 1000 mg/kg bw/day (the highest dose tested) for both males and females.

In the combined chronic and carcinogenicity study (2 years) in rat (0, 400, 2000, 7000, 15000 ppm), the NOAEL for males was considered to be 2000 ppm (105.1 mg/kg bw/day) due to the magnitudes of the decreased body weight and body weight gain (at 15000 ppm) and toxicological alterations in the liver including increased liver weights in combination with a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (both at ≥ 7000 ppm) and increased blood biochemistry parameters (total cholesterol and gamma-glutamyltransferase in males offered 15000 ppm).
For females, body weight and body weight gain was significantly decreased at ≥ 2000 ppm following 104 weeks of treatment. Toxicological alterations in the liver in females included increased liver weights (at ≥ 2000 ppm), a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (at ≥ 2000 ppm and at ≥ 7000 ppm, respectively) and increased blood biochemistry parameters (increased total cholesterol and increased gamma glutamyltransferase at ≥ 7000 ppm and at ≥ 15000 ppm, respectively). Therefore, the NOAEL for females was considered to be 400 ppm (26.7 mg/kg bw/day) for this study following 104 weeks of treatment.

Regarding the carcinogenic potential of mandestrobin, no increase of neoplastic findings exceeding the historical control range was observed in any organ of treated animals in the 2-year rat study, with exception of benign sex-cord stromal tumours in the ovary, which are discussed in detail in section *4.10 Carcinogenicity*.

In the 78 weeks feeding study in CD-1 mice (0, 700, 2000, and 7000 ppm), treatment with mandestrobin was well-tolerated. In the absence of adverse effects, the NOAEL for this study was

considered to be 7000 ppm (823.9 mg/kg bw/day for males and 994.0 mg/kg bw/day for females), the top dose tested, following 78 weeks of treatment.

Taking into consideration the cut off values for repeated dose toxicity for the oral route (for 90-day study → STOT RE 1: 10 mg/kg bw/d, STOT RE 2: 100 mg/kg bw/d) and for the dermal route of exposure (for 28-day study → STOT RE 1: 60 mg/kg bw/d, STOT RE 2: 600 mg/kg bw/d) and the nature of the observed effects in rat, mouse and dog studies, no classification and labelling for repeated dose toxicity is triggered for mandestrobin.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

4.8.1 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

Effects observed in the subchronic (oral and dermal) and chronic (oral) studies in rat, mouse and dog do not trigger classification and labelling of mandestrobin as STOT RE according to CLP Regulation (for details see section *4.7 Repeated dose toxicity*).

4.8.2 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

To get a systematic overview on the mandestrobin information relevant for the STOT RE classification, the following table with study-specific cut-off levels used by RAC, the study NOAELs and LOAELs, and the most critical/severe effects at LOAEL is presented.

Table 36: Summary table of repeated dose toxicity studies with mandestrobin and comparison with STOT RE criteria

Study	STOT RE1	STOT RE2	NOAEL and LOAEL	Adverse effects at LOAEL
Rat, 90-days (oral)	10	100	NOAEL: ♂ 54 mg/kg bw/day ♀ 320.1 mg/kg bw/day LOAEL: ♂ 282.6 mg/kg bw/day ♀ 788.5 mg/kg bw/day	Effects at LOAEL: ♂: ↑ absolute and relative liver weight ♀: ↑ absolute and relative liver weight; Hepatocellular hypertrophy; Follicular cell hypertrophy in the thyroid; ↑ Cholesterol levels
Mouse, 90-days (oral)	10	100	NOAEL: ♂ 807.3 mg/kg bw/day ♀ 1111.2 mg/kg bw/day	No treatment related adverse effects observed at the highest tested dose level
Dog, 90-days (oral)	10	100	NOAEL: ♂ 90.9 mg/kg bw/day ♀ 102.7 mg/kg bw/day LOAEL: ♂ 267.8 mg/kg bw/day ♀ 304.4 mg/kg bw/day	Effects at LOAEL: ↑ liver weight; Pigmentation of the liver; Centrilobular degeneration; ↑ alkaline phosphatase levels
Dog, 1-year (oral)	2.5	25	NOAEL: ♂ 19.2 mg/kg bw/day	Effects at LOAEL: ♂: hepatocyte hypertrophy,

			♀ 92.0 mg/kg bw/day LOAEL: ♂ 92.0 mg/kg bw/day ♀ 225.7 mg/kg bw/day	pigmentation, ↑ alkaline phosphatase levels ♀: ↑ rel liver weight, hepatocyte hypertrophy, Pigmentation, ↑ alkaline phosphatase levels
Rat, 28-days (dermal)	60	600	NOAEL: 1000 mg/kg bw/d	No treatment related effects observed at the highest tested dose level
Rat, 104 weeks (oral)	1.25	12.5	NOAEL: ♂ 105.1 mg/kg bw/d ♀ 26.7 mg/kg bw/d LOAEL: ♂ 375.6 mg/kg bw/d (7000 ppm) ♀ 135.2 mg/kg bw/d (2000 ppm)	Effects: ↓ body weight and bw gain (♂ at 15000 ppm, ♀at ≥ 2000 ppm) ↑ liver weight (♂ at ≥ 7000 ppm, ♀at ≥ 2000 ppm) ↑ hepatocellular hypertrophy (♂ at ≥ 7000 ppm, ♀at ≥ 2000 ppm) ↑ hepatocyte vacuolation (≥ 7000 ppm) ↑ total cholesterol (♂ at 15000 ppm, ♀at ≥ 7000 ppm) ↑ GGT (at 15000 ppm)
Mouse, 78 weeks (oral)	1.7	17	NOAEL: ♂ 823.9 mg/kg bw/d ♀ 994.0 mg/kg bw/d	Effects: No adverse effects of treatment at the highest dose tested

None of the repeated dose toxicity study with mandestrobin (i.e. subchronic oral and dermal and chronic oral studies in rat, mouse and dog) listed in the table above had a LOAEL value below the cut-off values for triggering classification for STOT RE according to CLP Regulation.

STOT RE classification may also be assigned on the basis of findings of significant or severe toxicity from reproductive toxicity studies. Evaluation of this data is outlined in section *4.11 Toxicity for reproduction*.
The only reproductive toxicity study with a LOAEL below the cut-off value for triggering classification for STOT RE (considering the cut-off value of 100 mg/kg bw/d for 90-day oral rat, which is a conservative approach as the administration period of parental animals was at least 16 weeks) is the Two-generation study in the rat (Matsuura, 2012) with a LOAEL of 60.19 mg/kg bw/d (1000 ppm) in female parental animals based on liver effects (weight increase, diffuse hypertrophy of hepatocytes) observed in the F0 and F1 generation.
Liver weights increased in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. Diffuse hypertrophy of the hepatocyte was observed in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. It is known that hepatocellular hypertrophy accompanied by increased liver weights is an adaptive change associated with induction of the hepatic microsomal drug metabolizing enzymes, and it is considered not to be adverse in the absence of histopathological damage indicative of hepatotoxicity and relevant clinical chemistry changes. In the 1000 ppm group, only hepatocellular hypertrophy and increased liver weights were observed in F0 and F1 females, without any other change. Therefore, the changes in the liver observed in females in the 1000 ppm group were considered to be an adaptive change, and of no toxicological significance, and thus do not warrant classification.

In conclusion, no effects were observed in the whole data package triggering classification and labelling of mandestrobin as STOT RE according to CLP Regulation.

4.8.3 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

Effects observed in the subchronic (oral and dermal) and chronic (oral) studies in rat, mouse and dog do not trigger classification and labelling of mandestrobin as STOT RE.

4.9 Germ cell mutagenicity (Mutagenicity)

Table 37: Summary table of relevant in vitro and in vivo mutagenicity studies

Method	Results	Remarks	Reference
***In vitro* studies**			
Bacterial reverse mutation (OECD 471)	negative (+/- S-9 mix)	*Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and *Escherichia coli* (WP2uvrA) Dose range: 9.77-5000 µg/plate (-S9) 39.1-5000 µg/plate (+S9)	Kitamoto, S.; 2010a
In vitro mammalian chromosomal aberration test (OECD 473)	negative (+/- S-9 mix)	Chinese hamster lung cells (CHL/IU) Dose range: 1.95-120 µg/mL (-S9) 25-150 µg/mL (+S9)	Kitamoto, S.; 2010b
In vitro Chinese hamster V79/HPRT locus gene mutation assay (OECD 476)	negative (+/- S-9 mix)	Chinese hamster V79 cells Dose range: 0.25-70.0 µg/mL (-S9) 8.0-172.0 µg/mL (+S9)	Wollny, H.E.; 2010
***In vivo* studies**			
Mouse micronucleus test (OECD 474)	negative	CD-1 mice 0, 500, 1000, 2000 mg/kg bw (suspended in Methylcellulose; single oral treatment)	Kitamoto, S.; 2010c

4.9.1 Non-human information

4.9.1.1 In vitro data

Bacterial assay for gene mutation

Mandestrobin was evaluated for its mutagenic potential by a reverse mutation test (Kitamoto 2010a) with four strains of *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and one strain of *Escherichia coli* (WP2*uvr*A). The test was conducted by the preincubation method in the presence and absence of metabolic activation (rat liver S9 mix).

Cytotoxicity (range-finding assay):

In a dose-finding assay the doses ranged from 4.88-5000 µg/plate. Cytotoxicity in strains TA100, TA1535 and TA1537 was observed at, and above, 313 µg/plate without S9 mix and at, and above, 1250 µg/plate with S9 mix. No cytotoxicity was seen at any dose for the other strains with or without S9 mix. Precipitation of the test compound was observed at and above 1250 µg/plate with and without S9 mix.

Mutation assay:
In the main assays (I and II), mandestrobin was tested in triplicate at doses ranging from 9.77-313 µg/plate for TA100, TA1535 and TA1537 without S9 mix, from 39.1-1250 µg/plate for TA100, TA1535, TA1537 with S9 mix, and from 156-5000 µg/plate for WP2*uvrA* and TA98 with and without S9 mix.

Table 38: Results of reverse mutation test in bacterial systems

Substance (µg/plate)	**S9-mix**	**Revertant colonies/plate (mean)**									
		TA100		**TA1535**		**WP2*uvr*A**		**TA98**		**TA1537**	
		Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II	Assay I	Assay II
0	-	85	82	8	8	23	27	21	24	10	14
9.77	-	92	81	8	10	NT	NT	NT	NT	7	9
19.5	-	86	86	9	8	NT	NT	NT	NT	10	12
39.1	-	85	84	8	12	NT	NT	NT	NT	11	10
78.1	-	91	86	8	9	NT	NT	NT	NT	11	10
156	-	87	93	9	9	25	17	25	23	11	11
313	-	65*	66*	5*	5*	27	25	27	21	6*	7*
625	-	NT	NT	NT	NT	28	23	23	22	NT	NT
1250†	-	NT	NT	NT	NT	23	26	23	24	NT	NT
2500†	-	NT	NT	NT	NT	22	25	19	22	NT	NT
5000†	-	NT	NT	NT	NT	19	22	16	20	NT	NT
PC	-	519	566	250	263	102	101	303	330	366	326
0	+	81	78	8	8	29	28	24	28	15	15
39.1	+	96	81	5	8	NT	NT	NT	NT	16	11
78.1	+	88	94	9	9	NT	NT	NT	NT	7	11
156	+	93	96	6	10	29	33	34	25	11	13
313	+	79	85	7	6	33	30	24	27	11	12
625	+	82*	72*	6*	8*	25	28	25	26	12*	13*
1250†	+	78*	74*	5*	9*	27	27	25	20	7*	11*
2500†	+	NT	NT	NT	NT	19	26	29	23	NT	NT
5000†	+	NT	NT	NT	NT	20	22	24	20	NT	NT
PC	+	701	611	208	184	516	503	207	197	103	110

PC positive control
NT not tested
* toxic effects observed
† precipitation

Mandestrobin was not mutagenic in the bacterial reverse mutation assay under the test conditions.

Test for clastogenicity in mammalian cells

Mandestrobin was evaluated for its clastogenic potential by an in vitro chromosomal aberration test in Chinese haster lung cells (CHL/IU) (Kitamoto 2010b).

Preliminary cytotoxicity assay:

Based on the solubility limit in DMSO, the highest concentration was set at 1000 μg/mL in the medium. Precipitates were seen in the medium at the beginning of treatment at and above concentrations of 125 μg/mL and at the end of treatment at and above concentrations of 500 μg/mL.

Marked growth inhibition was seen after exposure of CHL cells (+/- S9) to mandestrobin. The dose-response was steep: (i) without S9, from 48.2% growth at 62.5 μg/mL to 15.1% growth at 125 μg/mL for 6 hours treatment, and from 25.9% growth at 62.5 μg/mL to 3.3% growth at 125 μg/mL, for 24 hours treatment, and (ii) with S9, from 77.0% growth at 62.5 μg/mL to 42.0% at 125 μg/mL, to 1.6% at 250 μg/mL.

Cytogenicity and chromosome analysis:

In the initial 6 hour exposure assay, in the absence of S9 no precipitate was seen in the medium at any concentrations and because the growth rate at 80.0 μg/mL was not less than 50% a metaphase spread was not prepared in this test and the second trial was performed. In the second trial precipitates were seen in the medium at the beginning of treatment at concentrations of 120 μg/mL. Slides from 40, 60 and 80 μg/mL were analysed. A concentration of 80 μg/mL caused slightly greater than 50% cell growth inhibition, so this concentration and two lower concentrations were analysed. In the presence of S9, precipitates were seen in the medium at the beginning of treatment at and above concentrations of 125 μg/mL. Slides from 100, 125 and 150 μg/mL were analysed. A concentration of 150 μg/mL caused more than 50% cell growth inhibition so this concentration and two lower concentrations were analysed. The test compound induced no increase in the incidence of chromosomally aberrant cells (structural, numerical or polyploid) in any treatment groups with or without S9.

In a 24 hour exposure assay, CHL cells without S9 were treated with 5 doses, starting with 31.3 μg/mL and decreasing sequentially by a factor of 2. A concentration of 15.6 μg/mL caused more than 50% cell growth inhibition, so slides from 3.91, 7.81 and 15.6 μg/mL were analysed, and showed no increases in the incidence of structurally aberrant cells or polyploid cells. This study was considered confirmatory of the negative response seen in the absence of S9 after the 6-hour exposure.

In a repeat 6 hour exposure assay with S9 mix using exposures from 25 – 150 μg/mL, no increases in the incidence of structurally aberrant cells or polyploid cells was observed.

All the negative control cultures gave values of chromosome aberrations (structural aberration and polyploidy) within the expected range. Positive control chemicals MMC and CP displayed clear and expected increases in the incidence of cells with structural aberrations.

Table 39: Results of the in vitro chromosomal aberration test

Group	Dose	Rel growth	N	Structural Aberrations										Ishidate Judge-ment	Polypl
				No. of Aberrations								Cells with abs			
	(µg/mL)	(%)		gap	ctb	cte	csb	cse	Mul	Tot		(%)			(%)
										+G	-G	+G	-G		
6-Hour Exposure, without S9															
Control	0	100	200	0	1	0	0	0	0	1	1	0.5	0.5	-	0.0
S-2200 TG	40	56.6	200	2	1	0	0	0	0	3	1	1.5	0.5	-	2.5
	60	52.9	200	0	0	0	0	0	0	0	0	0.0	0.0	-	2.0
	80	43.7	200	0	3	0	0	0	0	3	3	1.5	1.5	-	1.5
MMC	0.06	75.0	200	6	23	35	0	1	1	75	69	26.0	24.0	+	0.0
6-Hour Exposure, with S9															
Control	0	100	200	0	0	0	0	0	0	0	0	0.0	0.0	-	0.5
S-2200 TG	100	62.9	200	1	2	6	0	0	0	9	8	2.0	1.5	-	2.0
	125 [p]	55.6	200	1	2	0	0	0	0	3	2	1.5	1.0	-	2.0
	150 [p]	31.8	200	3	4	5	0	0	0	12	9	4.5	3.5	-	3.0
CP	10	53.3	200	13	64	55	0	0	0	132	119	41.5	38.5	+	0.0
24-Hour Exposure, without S9															
Control	0	100	200	1	2	0	0	0	0	3	2	1.5	1.0	-	0.5
S-2200 TG	3.91	70.7	200	2	1	0	0	1	0	4	2	2.0	1.0	-	0.0
	7.81	65.1	200	3	4	1	0	0	0	8	5	4.0	2.5	-	0.0
	15.6	48.3	200	2	1	0	0	0	0	3	1	1.0	0.5	-	0.0
MMC	0.02	85.8	200	4	29	10	0	1	0	44	40	19.5	18.0	+	0.0
6-Hour Exposure, with S9, first repeat															
Control	0	100	200	2	0	0	0	0	0	2	0	1.0	0.0	-	0.0
S-2200 TG	100	77.1	200	1	0	1	0	0	0	2	1	1.0	0.5	-	0.5
	125 [p]	61.6	200	1	4	3	0	1	0	9	8	3.0	2.5	-	2.5
	150 [p]	46.2	200	1	4	0	0	0	0	5	4	2.5	2.0	-	1.5
CP	10	56.3	200	6	58	61	0	0	0	125	119	46.0	45.5	+	0.0

N – number of metaphases analysed
- Negative, + Positive, ± Marginal as determined by criteria of Ishidate
[p] – Precipitation observed at the beginning of the experiment
Rel growth – % of controls, cytotoxicity
ctb – chromatid break
cte – chromatid exchange
csb – chromosome break
cse – chromosome exchange
Mul – multiple aberrations (cells with more than 9 aberrations)
Tot – total aberrations
+G – aberrations including gaps; -G – aberrations excluding gaps
Polypl – polyploid cells and cells with endoreduplication

Mandestrobin has no potential to induce chromosomal aberrations in Chinese hamster lung cells in culture under the conditions tested.

Gene mutation assay with mammalian cell

Mandestrobin was evaluated for its mutagenic potential in mammalian cells by a gene mutation assay in Chinese hamster V79 cells in vitro (V70/HPRT) (Wollny 2010).

Preliminary cytotoxicity assay:

The tested concentrations were:
Without S9 mix, 4 hours treatment: 0, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0†, 500.0†, 1000.0† µg/mL;
Without S9 mix, 24 hours treatment: 0, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0†, 1000.0† µg/mL;
With S9 mix, 4 hours treatment: 0, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0†, 1000.0† µg/mL

(† precipitate formation)

Mandestrobin caused significant cytotoxicity. After 4 hour treatment cell survival was reduced to 16.3% at 15.6 µg/mL without S9 mix and to less than 10% at 250 µg/mL with S9 mix. After 24 hours of treatment cell survival was reduced to less than 10% at 62.5 µg/mL without S9.

Mutation assays:

Experiment I (4 hour treatment): Nine doses from 0.25 – 12.0 µg/mL were chosen for treatment without S9 mix, and the six treatments from 1.0 – 10.0 µg/mL were selected for evaluation. Six doses from 8.0 – 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 8.0 – 128.0 µg/mL were selected for evaluation. The maximum dose was selected based on cytotoxicity.

Experiment IA (4 hour treatment): Seven doses from 16.0 – 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 16.0 – 144.0 µg/mL were selected for evaluation. The maximum dose was selected based on cytotoxicity.

Experiment II (24 hour treatment without S9 mix and 4 hour treatment with S9 mix): Nine doses from 1.88 – 70.0 µg/mL were chosen for treatment without S9 mix, and five doses from 7.5 – 50.0 µg/mL were selected for evaluation. Seven doses from 16.0 – 172.0 µg/mL were chosen for treatment with S9 mix, and five doses from 16.0 – 144.0 µg/mL were selected for evaluation. The maximum dose was again selected based on cytotoxicity.

Negative and positive controls were within the expected historical range. No relevant and reproducible increase in mutant colony number/10^6 cells were noted in the main experiments up to the maximum concentration. Isolated increases of the mutation frequency, exceeding the threshold of three times the mutation frequency of the corresponding solvent control, occurred occasionally but were not reproduced in the parallel cultures under identical conditions, and were based on the relatively low solvent controls. Compared to the corresponding negative controls, the threshold was not exceeded. In addition, the mean values of mutation frequency between the first and the second cultures at 8.0 µg/mL (experiment I with metabolic activation), 32.0 µg/mL (experiment IA with metabolic activation) and 144.0 µg/mL (experiment II with metabolic activation) were below the threshold and were within the historical control range. Furthermore, linear regression analysis (least squares) found no significant dose-dependent trend of the mutation frequency indicated by a p value <0.05 in all of the experimental groups.

Table 40: Results of In Vitro Mammalian Gene Mutation (V79-HPRT)

Substance	**Dose**	**Relative cloning efficiency I - survival**		**Relative cloning efficiency II - viability**		**Mutant colonies per 10^6 cells**	
	(µg/mL)	Culture 1	Culture 2	Culture 1	Culture 2	Culture 1	Culture 2

Experiment I (without S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	20.1	12.9
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	22.7	9.3
Positive control (EMS)	150.0	90.8	89.2	82.5	92.8	168.2	84.2
Mandestrobin	0.25	93.4	96.4	#	#	#	#
	0.5	91.7	93.2	#	#	#	#
	1.0	90.7	87.6	109.0	91.6	15.0	7.7
	2.0	87.3	86.3	107.4	81.2	19.8	11.9
	4.0	65.6	83.2	100.2	103.8	24.8	6.0
	6.0	25.8	58.6	96.9	90.4	11.5	9.7
	8.0	5.9	42.4	99.3	92.5	13.3	7.8
	10.0	4.3	11.8	101.9	79.5	4.4	20.9
	12.0	##	##	##	##	##	##
Experiment I (with S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	7.8	23.2
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	11.4	7.9
Positive control (DMBA)	1.1	63.0	70.8	73.4	83.5	528.9	849.8
Mandestrobin	8.0	97.6	100.5	89.1	94.0	12.9	25.0
	16.0	94.7	97.8	82.2	90.9	9.0	13.6
	32.0	98.7	96.8	93.5	100.5	8.2	11.7
	64.0	96.3	96.1	111.0	98.4	21.0	13.3
	128.0	67.4	81.5	86.6	105.1	15.5	22.3
	172.0	0.1	0.2	##	##	##	##
Experiment IA (with S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	17.6	16.2
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	6.5	12.6
Positive control (DMBA)	1.1	75.0	80.0	69.6	80.7	761.8	827.8
Mandestrobin	16.0	91.8	99.7	97.7	92.2	9.7	9.6
	32.0	85.7	100.4	81.9	99.9	20.2	17.3
	64.0	99.9	101.7	85.8	88.3	8.7	29.4
	128.0	11.0	64.5	97.8	87.9	9.4	15.8
	144.0	2.8	7.2	82.7	85.0	2.7	5.1
	160.0	0.0	0.0	#	#	#	#
	172.0	0.0	0.0	#	#	#	#
Experiment II (without S9, 24 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	12.4	18.4
Solvent control (DMSO)	0	100.0	100.0	100.0	100.0	15.5	11.6
Positive control (EMS)	150.0	166.2	97.8	97.1	98.2	352.2	396.4
Mandestrobin	1.88	185.8	101.2	#	#	#	#
	3.75	104.3	97.3	#	#	#	#
	7.5	120.9	97.1	97.9	96.3	10.3	8.7
	15.0	176.4	94.3	101.6	98.1	12.7	6.4
	30.0	76.0	56.0	99.1	99.2	8.2	10.5
	40.0	46.0	35.3	98.2	95.5	11.7	11.4
	50.0	20.0	8.9	96.5	93.0	15.8	15.2
	60.0	0.0	0.0	##	##	##	##
	70.0	0.0	0.0	##	##	##	##
Experiment II (with S9, 4 hours treatment)							
Negative control	-	100.0	100.0	100.0	100.0	10.8	18.2
Solvent control	0	100.0	100.0	100.0	100.0	9.3	7.8

(DMSO)							
Positive control (DMBA)	1.1	38.3	41.4	97.8	76.6	675.2	816.8
Mandestrobin	16.0	98.8	105.2	103.1	98.8	10.0	18.8
	32.0	99.2	107.5	98.7	96.1	9.1	11.6
	64.0	98.3	104.3	100.3	93.9	13.6	8.8
	128.0	60.1	79.3	98.2	100.8	10.9	22.5
	144.0	10.9	27.5	102.7	94.4	6.3	38.0
	160.0	6.2	13.6	##	##	##	##
	172.0	0.0	0.0	##	##	##	##

Relative: as % of controls
[p] – Precipitate
- Culture was not continued, five higher concentrations were selected to be evaluated at the end of the experiment
- Culture was not continued due to strong toxic effects

In the *in vitro* mutagenicity study mandestrobin did not induce gene mutations at the HPRT locus in V79 cells.

4.9.1.2 In vivo data

Micronucleus test in mice after oral administration

For the micronucleus assay, groups of 5 male mice received a single gavage dose of 0, 500, 1000, or 2000 mg/kg mandestrobin and bone marrow smears prepared 24 hours later. Another group received 2000 mg/kg and smears were prepared at 48 hours. There was an additional cyclophosphamide (CP) positive control group (smears prepared at 24 hours), and two solvent (0.5% w/v aqueous methylcellulose) negative control groups (smears prepared at 24 and 48 hours).

In confirmation of the initial toxicity test, no mice died as a result of administration of mandestrobin and no abnormal signs were observed in the mandestrobin treated animals. There was no dose-related increase in micronuclei as a result of mandestrobin administration. The positive control showed appropriate increase in micronuclei formation to validate the sensitivity of the assay. There was no decrease in the PCE/(PCE+NCE) ratio after exposure to mandestrobin.

Although no change in the PCE/NCE ratio was found, a slight decrease of body weight gain at 2000 mg/kg bw in males in the range finding test indicates systemic absorption. In addition, sufficient exposure of bone marrow was confirmed in the single dose ADME studies in rats (Sumitomo Chemical Co. Ltd. Report No. ROM-0033). In the 5 mg/kg dose group, mandestrobin derived radioactivities in bone marrow were 234.1 (male) and 216.9 (female) ng equivalents/g at 0.5 hours post-dose. In 1000 mg/kg dose group, mandestrobin derived radioactivities in bone marrow were 7455 (male) and 3828 (female) ng equivalents/g at 8 hours post-dose. Both mandestrobin and its metabolites were detected in plasma at the timepoints stated above.

Table 41: Results of *In Vivo* Micronucleus Test

Treatment	**Dose (mg/kg)**	**Sampling time (hr)**	**Micronucleated PCE (%, mean ± SD)**	**PCE ratio (%, mean ± SD)**
Control	0	24	0.35 ± 0.158	52.0 ± 4.66
Mandestrobin	500		0.28 ± 0.057	54.7 ± 5.10
	1000		0.42 ± 0.211	55.9 ± 2.08

	2000		0.42 ± 0.160	57.1 ± 2.41
Cyclophosphamide	60		4.09 ± 0.783**	44.5 ± 5.18*
Control	0	48	0.29 ± 0.185	50.3 ± 3.91
Mandestrobin	2000		0.33 ± 0.120	51.4 ± 6.67
PCE - polychromatic erythrocytes NCE – normochromatic erythrocytes *p < 0.05; **p ≤ 0.01 Micronuclei: 2000 PCE were examined from each animal PCE ratio: PCE/(PCE+NCE), 1000 erythrocytes examined from each animal				

Mandestrobin did not cause an increase in the number of micronucleated PCE in male mice.

4.9.2 Human information

No human information.

4.9.3 Other relevant information

No other relevant information.

4.9.4 Summary and discussion of mutagenicity

Mandestrobin was tested in a sufficient range of *in vitro* and *in vivo* mutagenicity assays measuring different mutagenic endpoints such as gene mutation in bacterial and mammalian cells, clastogenicity *in vitro* as well as an *in vivo* micronucleus test in mice.

The results of all these studies mentioned show that **no mutagenic potential** attributed to mandestrobin under the test conditions used can be derived.

4.9.5 Comparison with criteria

No genotoxic effects were observed in studies with mandestrobin, neither in *in vivo* nor *in vitro* studies (according to CLP).

4.9.6 Conclusions on classification and labelling

There is no evidence of genotoxic potential of mandestrobin, therefore, no classification and labelling is proposed.

4.10 Carcinogenicity

Table 42: Summary table of relevant carcinogenicity studies

Method	Dose levels / NOAEL / Effects	Remarks	Reference
Rat oral via diet, 104 weeks (OECD 453)	0, 400, 2000, 7000, 15000 ppm equivalent to 0, 21.0, 105.1, 375.6 and 804.3 mg/kg bw/day (males) and 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw/d (females) <u>NOAEL</u>: 105.1 mg/kg bw/d (males) 26.7 mg/kg bw/d (females) <u>Effects</u>: ↓ body weight and bw gain (♂ at 15000 ppm, ♀at ≥ 2000 ppm) ↑ liver weight (♂ at ≥ 7000 ppm, ♀at ≥ 2000 ppm) ↑ hepatocellular hypertrophy (♂ at ≥ 7000 ppm, ♀at ≥ 2000 ppm) ↑ hepatocyte vacuolation (≥ 7000 ppm) ↑ total cholesterol (♂ at 15000 ppm, ♀at ≥ 7000 ppm) ↑ GGT (at 15000 ppm)	Crl:WI(Han) rats Purity: 93.4%	Beck, W.; 2012b
Mouse oral via diet, 78 weeks (OECD 451)	0, 700, 2000, 7000 ppm equivalent to 0, 82.5, 238.8 and 823.9 mg/kg bw/d in males and 0, 99.2, 280.3 and 994.0 mg/kg bw/d in females <u>NOAEL</u>: 823.9 mg/kg bw/d (males) 994.0 mg/kg bw/d (females) <u>Effects</u>: No adverse effects of treatment at the highest dose tested	Crl:CD1(ICR) mice Purity: 93.4%	Beck, W.; 2012c

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Rats

Reference:	S-2200 Technical Grade: 104 Week Oral (Dietary) Administration Combined Toxicity/Carcinogenicity Study in the Rat
Author(s), year:	Beck, W.; 2012b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0072
Guideline(s):	OECD 453 (1981), EPA OPPTS 870.4300 (1998), Japanese MAFF 12 Nohsan 8147 (2-1-16) (2001), Reg. (EC) No. 440/2008, B33 (2008)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	Thyroid was not weighed although it is one of the organs which is mandatory to investigate according to OECD 453
Validity:	Yes

MATERIAL AND METHODS:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date: 21 November 2011 (after completion of treatment)
Vehicle:	None. Test material was mixed directly into diet.
Test animals:	
Species:	Rat
Strain:	Crl:WI(Han)
Age:	approximately 6 weeks at the start of treatment
Weight at dosing:	140.6 to 225.0 g for males and 113.0 to 178.6 g for females
Source:	Charles River Laboratories, Margate, UK
Diet:	Finely ground SQC Rat and Mouse Maintenance Diet No 1 (Special Diets Services Ltd, Witham, UK) *ad libitum*

The purpose of this study was to assess the chronic toxicity as well as carcinogenic potential of mandestrobin (S-2200 TG) after dietary administration to rats for 52 weeks (chronic toxicity cohort; 20 animals per sex and dose group) and 104 weeks (carcinogenicity cohort; 50 animals per sex and dose group).

Animal assignment and treatment:

70 animals per sex were assigned to each treatment group using a total randomisation procedure. 20 animals per sex (i.e. chronic toxicity cohort) in each treatment group were used for interim sacrifice and examination at 52 weeks. Following the first full weighing (day -7), group mean body weights and standard deviations were calculated and inspected to ensure there

were no unacceptable differences between the groups. The rats received S-2200 TG in the diet at concentrations of 0 (control), 400, 2000, 7000 and 15000 ppm.

Diet preparation and analysis:

Formulations were prepared weekly. The test article was formulated as a diet mix in SQC Rat and Mouse Maintenance Diet No 1 (ground fine).

Formulations were analysed for their test article content and homogeneity using High Performance Liquid Chromatography with Ultra Violet detection (HPLC-UV), based on a method supplied by the Sponsor, and was validated in Covance Study Number 8201819.

The test article was demonstrated to be stable in diet at 100 and 20000 ppm when stored for 15 days at ambient temperature (Covance Study Number 8201819).

Table 43: Group mean compound intakes (mg/kg bw/d) following 52 (chronic toxicity cohort) and 104 (carcinogenicity cohort) weeks of treatment

Group	**Dose level (ppm)**	**Mean compound consumption (mg/kg bw/d)**			
		Chronic toxicity cohort		**Carcinogenicity cohort**	
		Male	**Female**	**Male**	**Female**
1 (control)	0	-	-	-	-
2 (low)	400	25.5	31.3	21.0	26.7
3 (intermediate I)	2000	130.3	151.4	105.1	135.2
4 (intermediate II)	7000	448.8	535.3	375.6	475.0
5 (high)	15000	991.8	1138.9	804.3	1016.2

Clinical observations:

All animals were observed at the beginning and the end of the working day to ensure they were in good health. All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a detailed physical examination including palpation for tissue masses at weekly intervals.

Body weight, food consumption and compound consumption:

Individual body weights were recorded on Day -7, once weekly from Day 1 (before dosing) to Week 16, once every 4 weeks thereafter and on the day of (prior to) necropsy. The amount of food consumed by each cage of animals was determined weekly from Week -1 to Week 16 and then on one week in every four thereafter. Consumption was calculated as g/animal/week.

Compound consumption was calculated once weekly from Week 1 to Week 16 and on one week in every four thereafter. Weekly compound consumption was calculated as mg/kg bw/day and also as an average consumption over the entire treatment period (mg/kg bw/day).

Functional observation battery:

All animals of the chronic toxicity cohorts were subjected to a battery of behavioural tests and observations before initiation of treatment and at once weekly intervals thereafter. In Week 51, an assessment was made of sensory reactivity to stimuli, grip strength and motor activity.

Where possible, the observations were performed at the same time on each occasion. All animals were observed for the following parameters:

- Home cage observations: Posture, activity, gait, tremor, convulsions, excessive vocalisation, arousal upon opening cage.
- Handling observations: Ease of removal from cage, ease of handling, excessive vocalisation, tremor, convulsions, palpebral closure, exophthalmus, lacrimation, lacrimation type, salivation, respiration, piloerection, fur appearance, other.
- Open field observations: Latency to first step, posture, arousal, circling, gait type, gait type severity, stereotypy, tremor, convulsions, other.
  And additionally in Week 51 only: Approach response, touch response, tail pinch, air righting ability, pupillary response, corneal tactile reflex test, auditory startle response, hindlimb foot splay, forelimb and hindlimb grip strength.

Ophthalmoscopy:

Investigations were performed on all animals of the chronic toxicity cohort pre-treatment and on the control and high dose group of the chronic toxicity cohort in Week 50. A mydriatic agent was instilled into the eyes before examinations.

Haematology, clinical chemistry and urinalysis:

Haematology:

Blood samples were withdrawn from the chronic toxicity cohort animals in Weeks 13, 26 and 52 and from the carcinogenicity cohort animals in Weeks 78 and 104. Samples were collected from the lateral caudal vein after an overnight period without food (fasting performed only for chronic toxicity cohort). Blood samples from decedents were taken from the abdominal aorta at necropsy, where possible.

For the chronic toxicity cohort, the following parameters were determined on blood taken into EDTA anticoagulant: haemoglobin concentration (Hb), red blood cell count, packed cell volume (PCV), absolute reticulocytes, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelet count, total and differential white blood cell count.

For the carcinogenicity cohort, the following parameters were determined on blood taken into EDTA anticoagulant: total and differential white cell count.

In addition, the following parameters were determined for the chronic toxicity cohort on plasma derived from whole blood taken into trisodium citrate anticoagulant: prothrombin time (PT) and activated partial thromboplastin time (APTT).

A blood smear was routinely prepared but only examined where an assessment of cell morphology was useful to support or clarify abnormalities identified by the automated analyser. Bone marrow smears were prepared at necropsy. They were fixed in methanol but not examined.

Clinical chemistry:

The following parameters were determined for the chronic toxicity cohort on plasma derived from whole blood collected into lithium heparin anticoagulant: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), sodium (Na), calcium (Ca), potassium (K), chloride (Cl), inorganic phosphorus (P), total protein, albumin, globulin, albumin/globulin ratio, triglycerides, total cholesterol, total bilirubin, glucose, urea, creatinine.

Urinalysis:

Urine samples were collected during a six-hour day time period from the chronic toxicity cohort in Week 12, 25 and 51. Food and water were removed during collection.

The following parameters were determined:

Volume (measured by weight, reported in mL; 1 g considered to be equivalent to 1 mL), colour, turbidity, specific gravity, pH*, protein*, glucose*, ketones*, urobilinogen*, bilirubin*, blood*, microscopy of sediment.

*determined semi-quantitatively

Sacrifice and pathology:

All animals, including the decedents, were subjected to necropsy.

The scheduled necropsies were performed after an overnight period without food. Where possible, they were carried out in replicate order. Each animal was given an intraperitoneal overdose of sodium pentobarbitone. Once a suitable deep plane of anaesthesia was established, the animal was exsanguinated by the severing of major blood vessels. A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded.

The following tissues from all animals were preserved in the appropriate fixative.

Table 44: Tissues preserved in appropriate fixative*; W = weighed; E = processed and examined microscopically

Adrenals	W	E	Oesophagus		E
Animal identification			Optic nerves		E
Aorta		E	Ovaries	W	E
Bone marrow smear (femur) (a) (d)			Pancreas		E
Brain	W	E	Pituitary		E
Caecum		E	Prostate		E
Colon		E	Rectum		E
Duodenum		E	Salivary glands – mandibular, sublingual, parotid		E
Eyes (b)		E	Sciatic nerves		E
Femur with bone marrow and articular surface		E	Seminal vesicles		E
Gross lesions		E	Skin and subcutaneous tissue		E
Harderian glands		E	Spinal cord – cervical		E
Head			Spinal cord – lumbar		E
Heart	W	E	Spinal cord thoracic		E
Ileum		E	Spleen	W	E

Jejunum		E	Sternum with bone marrow		E
Kidney	W	E	Stomach		E
Lacrimal glands			Testes and epididymides (c)	W	E
Larynx		E	Thymus		E
Liver	W	E	Thyroids and parathyroids		E
Lungs with mainstem bronchi and bronchioles		E	Tissue masses		E
Mammary		E	Tongue		
Mandibular lymph nodes		E	Trachea		E
Mesenteric lymph nodes		E	Trachea bifurcation		
Muscle (quadriceps)		E	Urinary bladder		E
Nares (e)		E	Uterus including cervix #	W	E
Nasal cavity (e)		E	Vagina		E
Nasopharynx (e)		E			

* Fixative = neutral-buffered 10% formalin except where indicated by:

a – methanol, b - Davidson's fluid, c - Bouin's fixative

d – Bone marrow smears were prepared at necropsy. They were fixed in methanol but not examined.

e – preserved with the head *in situ*

Bone designated for histopathological examination was decalcified using Kristenson's fluid.

Female uterus weights were captured with the oviducts attached to enable more accurate comparison of study data with background data.

Organ Weights:

Animals were weighed before necropsy. The organs denoted by "W" in the table (tissue list) above were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together. Organs from all animals of the chronic toxicity cohort and organs from the first 10 animals/sex/group of the carcinogenicity cohort that survived to the scheduled terminal kill were weighed. Due to the presence of lesions, organ weights from animals 58M (400 ppm), 154M (7000 ppm) and 214M (15000 ppm) were excluded and weights were captured from a different animal of the same group and sex. It must be mentioned that the thyroid weight was not recorded as detailed in the updated OECD guideline No. 453 from 07 September 2009. However, pathological examination was conducted. Therefore, this omission is considered not to have affected the outcome or integrity of the study.

Histopathology:

The following tissues were embedded in paraffin wax BP (block stage), sectioned at 5 µm and stained with haematoxylin and eosin:

Group 1 (control) Group 5 (15000 ppm) and decedents (all groups): All tissues denoted by "E" in the table (tissue list) above. The following tissues were examined in Groups 2 (400 ppm), 3 (2000 ppm) and 4 (7000 ppm) of the chronic toxicity cohort: Liver, thyroid, kidney (males only), gross lesions and tissue masses. The following tissues were examined in Groups 2, 3 and 4 of the carcinogenicity cohort: Liver, thyroid, kidney, ovary (females only), gross lesions and tissue masses.

Sectioned tissues were examined microscopically by the Study Pathologist.

Statistics:

Analysis of in-life and organ weight parameters:

The control group (Group 1) was taken as the baseline group with which the treated groups (Groups 2, 3, 4, 5) were compared. Body weights, body weight gains, food consumption (carcinogenicity cohort), necropsy body weights, organ weights, organ to necropsy body weight ratios, functional observational battery, locomotor activity, haematology, blood biochemistry and urine analysis variables were analysed using one-way analysis of variance (ANOVA), separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with the control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Selected haematology variables (monocytes, eosinophils, basophils and large unstained cells), clinical pathology parameters with values above or below the limit of the assay and functional observational battery variables with less than five distinct values were analysed using the non-parametric methods described above.

Food consumption (chronic toxicity cohort) was analysed using two-way analysis of variance (ANOVA). Levene's test for equality of variances across groups, between sexes and for any interaction was performed and where these tests showed no evidence of heterogeneity ($p \geq 0.01$ for all 3 tests), pairwise comparisons with control were made, for each sex separately, using Dunnett's test. For each sex, a linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Analysis of macroscopic findings:

All macroscopic finding data for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. Separate analyses were performed for the following: (i) Terminal kill toxicity animals, (ii) Decedent carcinogenicity animals, (iii) Terminal kill carcinogenicity animals and (iv) All carcinogenicity animals.

For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher’s Exact test.

Analysis of microscopic findings:

All microscopic finding data for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk. Separate analyses were performed for the following: (i) Terminal kill toxicity animals, (ii) Decedent carcinogenicity animals, (iii) Terminal kill carcinogenicity animals and (iv) All carcinogenicity animals.

For each microscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test. For each microscopic finding of interest, comparisons were made between the incidence in the treated group and that in the control group using the Wilcoxon-Mann-Whitney rank sum test.

Analysis of tumour data:

All tumour data for all tissues examined were supplied for analysis. Male and female data were analysed separately. For groups where all tissues were protocolled to be examined, the numbers of tumour bearing animals were analysed for tumour types found in at least three animals of the given sex. Tumours of similar histogenic origin were merged. Permutational tests for both an increasing and a decreasing dose response were performed across the groups using the dose levels as weighting coefficients, in accordance with the IARC annex. One directional pairwise tests of the treated groups against the control group were also performed.

Non-fatal tumours were analysed using fixed intervals of 1 to 52 weeks, 53 to 78 weeks, 79 to 105 weeks, the interim kill phase and the terminal kill phase. The fatal and non-fatal results were combined in accordance with the IARC annex. At the request of the Study Director, two separate analyses were performed; all animals and carcinogenicity animals only.

FINDINGS:

Mortality and clinical observations:

No treatment-related effect on mortality was detected. The numbers of decedents per group and the percentage of survival are detailed in Tables 45 and 46.

There were no clinical signs in the animals of chronic toxicity cohort or the carcinogenicity cohort that could be attributed to S-2200 TG.

Body weight, body weight gain and food consumption:

Chronic toxicity cohort:

Males offered 15000 ppm had significantly lower body weights than controls at all measured timepoints during the first 24 weeks of the study, which reflected the significant reduction in body weight gain from start to Week 13 (15.0%). Suppression of body weight was also statistically significant at Week 40 (8.1%). These changes are considered to be toxicologically significant.

For females offered 15000 ppm, body weights were lower than control at all measurement intervals, although attaining statistical significance only at Week 52. When compared with control, significant decreases in bw gain were noted from start to Week 13 (9.8%), from Week 28 to 52 (52.6%) and from start to Week 52 (16.4%). In consideration of the findings in the carcinogenicity cohort, the lower body weights in females dosed with 15000 ppm are considered to be toxicologically significant.

In males offered 7000 ppm and 15000 ppm, food consumption was lower than control (4.5% and 4.3%, respectively) over the 52 weeks of treatment, although the reduced intakes were not statistically significant. The lower consumption for males offered 7000 ppm is considered to be not toxicologically relevant based on the lack of a similar effect in the carcinogenicity cohort over

this duration and on the lack of a clear alteration in body weight. Females offered 15000 ppm consumed 7.5% less diet than control between Weeks 1 and 52 which was statistically significant in the pairwise comparisons at the $p < 0.05$ level.

All other changes were not considered to be toxicologically relevant based on the lack of temporal consistency and/or dose response relationship. Statistically significant increases in body weight, body weight gain and food consumption in males offered 400 ppm are considered to be incidental as there were no similar findings in the carcinogenicity cohort and in females of both cohorts.

Carcinogenicity cohort:

Significant suppression of body weights was seen from Week 2 onwards for males offered 15000 ppm. A decrease in body weight gain was significant at most of the selected periods culminating in an overall (start to Week 104) mean reduction of 17.6%, when compared with control.

With increasing dose, significant differences from control in female body weights were observed more rapidly. Females offered 2000 ppm had significantly decreased body weights at Week 68 and at all measurement intervals from Week 76 onwards. Differences were significant for females offered 7000 ppm at Week 24 and at all measurement intervals from Week 40 onwards, and for females offered 15000 ppm at Week 6 and from Week 11 onwards. Statistically significant decreases in overall body weight gains (start to Week 104) were observed for females offered 2000, 7000 and 15000 ppm (18.1%, 18.2% and 32.8%, respectively), when compared with control.

No statistically significant differences in food consumption were observed in the carcinogenicity cohort, when compared pairwise with control. However, for animals offered 15000 ppm intakes were consistently lower resulting in overall decreases of 4.4% and 4.8% for males and females, respectively.

Functional observational battery:

There were no findings from any assessment indicative of any neurotoxicological effect of the test formulations.

Ophthalmoscopy:

Ophthalmoscopic examinations performed in Week 50 in the control and high dose groups of the chronic toxicity cohort did not reveal any treatment related effects.

Haematology, clinical chemistry and urine analysis:

Haematology – chronic toxicity cohort:

The decreased haemoglobin concentrations observed in animals offered 15000 ppm were considered to be treatment-related based on temporal consistency. However, these alterations from control were generally small and values were within historical control ranges, where available (historic control data are only available for Week 13 and 26, not for Week 52). In addition, no findings were observed in related endpoints such as red blood cells, reticulocytes or morphological changes in blood forming organs. Therefore, these decreases are considered not to be of toxicological significance.

All other statistically significant differences between control and treated groups were consistent with normal variability, small, inconsistent between the sexes, lacking temporal consistency and/or unrelated to dose and, therefore, considered not to be treatment-related.

All statistically significant haematological findings observed in Week 52 are detailed in Table 45.

Haematology – carcinogenicity cohort:

In the carcinogenicity cohort only a total and differential white blood cell count was performed after 78 and 104 weeks of treatment. There were no inter-group differences in the haematological composition of the blood samples that could be attributed to treatment. All statistically significant differences between control and treated groups were small, inconsistent between the sexes, lacking temporal consistency and/or unrelated to dose and, therefore, considered to be of no toxicological importance.

A small number of control and treated animals killed during the treatment period or surviving to termination had elevated white blood cell counts generally due to increased neutrophils and/or lymphocytes, which generally correlated microscopically with the presence of haemolymphoreticular tumour.

All statistically significant haematological findings observed in Week 104 are detailed in Table 46.

Clinical chemistry: (chronic toxicity cohort only)

Gamma-glutamyltransferase was statistically significantly increased in both sexes offered 15000 ppm at Week 13 (M: 1.5-fold, F: 2-fold), at Week 26 (M: 2.5-fold, F: 1.5-fold) and at Week 52 (M: 4-fold, F: 3-fold) when compared with controls.

Total cholesterol was higher at Week 13 in animals offered 7000 ppm (M: 29%, F: 38%) and 15000 ppm (M: 47%, F: 56%), at Week 26 in females offered 7000 ppm and 15000 ppm (50% and 65%, respectively) and at Week 52 in females offered 7000 ppm (55%) and in both sexes offered 15000 ppm (M: 26%, F: 50%), when compared with controls. Therefore, the consistent increases seen in females offered 7000 ppm and in both sexes offered 15000 ppm were considered to be toxicologically relevant. As no disturbances were observed on subsequent occasions, the change in males offered 7000 ppm at Week 13 was considered not to be toxicologically significant.

All other statistically significant differences between control and treated groups were consistent with normal variability (i.e. within historic control ranges which, however, are only available for Week 13 and 26), small, inconsistent between the sexes, lacking temporal consistency and/or unrelated to dose and, therefore, considered incidental.

All statistically significant findings observed in Week 52 are detailed in Table 45.

Urinalysis: (chronic toxicity cohort only)

There were no obvious differences in urine analysis test results to indicate an effect of treatment.

The mean volume of urine voided by males offered 400 ppm at Week 12 and 51 and by males offered 15000 ppm at Week 51 was statistically increased when compared with control. Quantitative measurements indicated that excretion was approximately 64% to 71% higher. However, as there were no similar effects in females or overt differences in Week 25, these

changes are considered to be incidental and not related to treatment. Increased urine output correlated with a decrease in specific gravity.

Sacrifice and pathology - organ weights:

Chronic toxicity cohort:

There were treatment-related changes in liver, kidney and brain weights, when compared with controls.

Increased liver weights were notable in males offered ≥ 400 ppm and in females offered ≥ 2000 ppm. In view of the associated changes in the clinical chemistry and pathological investigations, the statistically significant alterations observed in both sexes offered 7000 ppm and 15000 ppm were considered to be toxicologically significant.

A statistically significant increase in the relative kidney weights was seen in males offered 15000 ppm. A statistically significant decrease in unadjusted brain weight was seen for males offered 15000 ppm (-5%). The relative brain weight was increased for females offered 15000 ppm. Changes in kidney and brain weight were considered not to be toxicologically significant as there were no macroscopic or microscopic correlates, and these changes may be related to body weight suppression.

When compared with control, mean relative ovary weights were statistically significantly increased (+26%) in females offered 15000 ppm. This finding is considered to be incidental as there was no similar difference seen for the carcinogenicity cohort at terminal kill and it was not associated with any pathological correlates.

All other organ weight (and/or organ weight ratio) changes were considered not to be biologically significant as they were either small in magnitude, not dose-dependent, inconsistent between the sexes, spontaneous background changes due to normal inter-animal variability and/or lacked a histopathological correlate.

Carcinogenicity cohort:

Increased liver weights were notable in males offered ≥ 7000 ppm and in females offered ≥ 2000 ppm. In view of the associated changes in the clinical chemistry and pathological investigations, the alterations observed in both sexes offered 7000 ppm and 15000 ppm were considered to be toxicologically significant.

All other organ weight (and/or organ weight ratio) changes were considered not to be biologically relevant as they were either small in magnitude, not dose-dependent, inconsistent between the sexes, spontaneous background changes due to normal inter-animal variability and/or lacked a histopathological correlate.

Sacrifice and pathology – macroscopic findings:

Chronic toxicity cohort:

Most tissues were macroscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in animals of this strain and age. However, large liver was recorded in males offered 15000 ppm with a statistically significant incidence. Other statistically significant differences between groups for the macroscopic findings such as mottled liver and red

mandibular lymph node, which correlated microscopically with agonal congestion/haemorrhage, were sporadic and considered to not be related to dose or test article toxicity.

Carcinogenicity cohort:

Most tissues were macroscopically unremarkable and the findings seen were generally consistent with the usual pattern of findings in animals of this strain and age. Large liver was variably recorded in males offered 7000 or 15000 ppm, although not statistically significant, which generally correlated with findings seen microscopically. Statistically significant differences between groups for the macroscopic findings noted in decedents and at the terminal kill, such as dark liver which correlated with agonal congestion/haemorrhage, were considered to be secondary and not related to test article toxicity.

Sacrifice and pathology – microscopic findings:

Chronic toxicity cohort:

Microscopic findings were generally infrequent, of a minor nature and consistent with the usual pattern of findings in animals of this strain and age. However, there were findings in the liver and thyroid in males and females, associated with treatment with the test article.

In the liver, there was a statistically significant increase ($p < 0.0001$) in the incidence and severity of hepatocellular eosinophilia/hypertrophy in animals offered 7000 or 15000 ppm, characterised by enlarged hepatocytes with increased amounts of eosinophilic cytoplasm. This was considered to be an adaptive change associated with test article metabolism. There was also a minor increase in hepatocyte vacuolation in males offered 15000 ppm (not statistically significant), characterised by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes. The toxicological significance of this change in this study is not clear.

In the thyroid, there was a statistically significant increase in the incidence and in the severity of follicular cell hypertrophy in animals offered 7000 or 15000 ppm. Follicular cell hypertrophy was characterised by follicles with columnar epithelium with increased amounts of apical cytoplasm, with or without a decrease in follicular colloid. This is generally considered to be an adaptive change due to increased thyroid hormone metabolism in the liver and is commonly associated with liver cell hypertrophy.

In the kidney, hyaline droplets were observed in males offered ≥ 400 ppm, but the incidences were not notably different from that of control males. Hyaline droplets were characterised by small, eosinophilic cytoplasmic inclusions within proximal convoluted tubule epithelium. Hyaline ($\alpha_{2\mu}$ globulin) droplets in the proximal tubular epithelial cells present a common response of the male rat to xenobiotics by a mechanism that is not relevant to humans.

Other statistically significant differences between dose groups were considered to be incidental and not related to test article toxicity, as they represented recognised background, age-related, non-specific or single-sex findings, decreases in finding incidences, or random occurrences in intermediate dose groups, without association with treatment.

Carcinogenicity cohort – non-neoplastic findings:

Microscopic non-neoplastic findings were generally infrequent, of a minor nature and considered to be within normal background levels in animals of this strain and age. However, in treated animals, there were treatment-related findings in the liver, thyroid gland, kidney and ovary.

In the liver, there was an increase in hepatocyte eosinophilia/hypertrophy in animals offered 2000 ppm and above, characterised by enlarged hepatocytes with increased amounts of pale eosinophilic cytoplasm. Increases in hepatocyte eosinophilia/hypertrophy were statistically significant in the liver of males offered 7000 or 15000 ppm and in females offered 2000 ppm and above. This was considered to be an adaptive change associated with test article metabolism. There was no associated increase in hepatic tumours noted in the study.

There was also a statistically significant increase in hepatocyte vacuolation in treated animals offered 7000 or 15000 ppm, characterised by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes. However, the toxicological significance of this change in this study is unknown.

In treated females, and to a lesser extent in males, there was an increase in bile duct hyperplasia. This finding was considered not to be toxicologically relevant as it was not observed in a dose-dependent manner and there was no associated bile duct epithelial degeneration or necrosis, fibrosis nor progression to bile duct neoplasia.

In the thyroid gland, there was an increase in follicular cell hypertrophy in males, and to a lesser extent in females, offered 15000 ppm; males and females in other dose groups were similar to controls. Thyroid follicular cell hypertrophy showed a statistically significant increase in males offered 15000 ppm. Follicular cell hypertrophy was characterised by follicles with columnar epithelium with increased amounts of apical cytoplasm, with or without a decrease in follicular colloid. This increase in follicular cell hypertrophy was not accompanied by an increase in thyroid follicular cell tumours and is known to be associated with liver hepatocellular hypertrophy. This is generally considered to be an adaptive change due to increased thyroid hormone metabolism in the liver.

In the kidney, there was an increase in papillary and cortico-medullary mineralisation in females offered 2000 ppm and above, and in pelvic mineralisation in females offered 7000 or 15000 ppm, though not necessarily statistically significant. Treated males were similar to controls. Papillary mineralisation was characterised by small amounts of dark basophilic mineral within the renal papillary tubules. Pelvic mineralisation was characterised by similar material within the renal pelvis or pelvic epithelium. Cortico-medullary mineralisation was characterised by small amounts of dark, basophilic material within the tubules and tubular epithelium at the cortico-medullary junction. This overall increase in mineralisation in some treated animals was considered to be associated with renal excretion of the test article and its metabolites, and not direct test article toxicity.

In the ovary, there was a minor increase in sex-cord stromal hyperplasia in females offered the test material although not in a dose-dependent manner and also without statistical significance when compared to controls. Sex-cord stromal hyperplasia was characterised by a hyperplastic lesion composed of any mixture of granulosa, theca, luteal or Sertoli cells within the ovarian parenchyma with minimal involvement of the surface epithelium and was considered to be part of a continuum of change, associated with the development of sex-cord stromal tumours. The incidence in treated animals was within current historical background data (range: 2-48%). In the background data, sex-cord stromal hyperplasia may be observed frequently in aged animals.

There was a statistically significant increase in oligospermia in the epididymis of males offered 15000 ppm; however, these findings were primarily noted unilaterally. They were considered to

be age-related changes due to their predominantly unilateral nature and in the absence of other male reproductive tract findings and therefore not associated with generalised hormonal disruption or test-article toxicity.

There were no other microscopic non-neoplastic findings considered to be related to treatment with S-2200 TG, since these were generally infrequent, of a minor nature and considered to be within normal background levels in animals of this strain and age.

Carcinogenicity cohort – neoplastic findings:

Microscopic neoplastic findings in controls and treated animals were generally consistent with the usual pattern of neoplasms in rats of this strain and age. However, there was an increase in sex-cord stromal tumours in the ovary of females offered 7000 or 15000 ppm. This was not statistically significant when compared with controls by pair-wise analysis. However, for tests of increasing dose response, benign sex cord stromal tumours in the ovary of females were statistically significant in the carcinogenicity cohort when compared to control animals ($p = 0.005$).

The increased incidence of sex-cord stromal tumour in the ovary exceeded the Covance, Harrogate historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that also the current control animals (incidence 2/50 = 4%) exceeded the historical control range for sex-cord stromal tumours. The tumours occurred at dose levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded. Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. Since there is no interaction of the test article with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays (see section 4.12.1.3 Specific investigations: other studies, Ovary issues, *In vitro* Steroidogenesis Assay of S-2200TG in H295R Cells (Kubo, H.; 2012) and Evaluation of Effects of S-2200 TG and its Metabolites on Human Estrogen Receptor alpha and Human Androgen Receptor Using *in vitro* Reporter Gene Assays (Suzuki, N.; 2012)), no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of S-2200 TG or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay (see next study), the number of tumours in any tissue was not increased by exposure to S-2200 TG. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

There was a reduction in the incidence of benign mammary fibroadenomas in females offered 15000 ppm and also a statistically significant reduction in the incidence of pituitary tumours (adenoma and carcinoma) in females offered 15000 ppm. This correlates with the reduction in fatal tumours observed in female decedents offered 15000 ppm.

There was also a minor increase in interstitial cell adenomas in the testis of males offered 15000 ppm. However, this was within historical control data at this time (range 0-6%), so it was considered to be a chance effect in the absence of any increase in hyperplastic lesions in this cell type or other evidence of hormonal effects within the male reproductive tract and not due to test article toxicity.

Table 45: Key results for the combined chronic toxicity/carcinogenicity study in rats – Chronic toxicity cohort

	Males					Females				
Diet concentration (ppm)	**0**	**400**	**2000**	**7000**	**15000**	**0**	**400**	**2000**	**7000**	**15000**
S-2200 intake (mg/kg bw/day)										
	0	25.5	130.3	448.8	991.8	0	31.3	151.4	535.3	1138.9
Mortality[#]										
	1/20	1/20	1/20	0/20	0/20	0/20	0/20	1/20	1/20	0/20
Survival %										
	95	95	95	100	100	100	100	95	95	100
Body weight (g)										
Week 0	184.7	189.1	182.8	180.1	180.0	140.2	140.9	143.5	141.0	138.7
% change from control		2.4	-1.0	-2.5	-2.5		0.5	2.4	0.6	-1.1
Week 13	370.6	394.6	359.2	348.6	**338.0****	227.8	220.7	225.3	227.5	217.7
% change from control		6.5	-3.1	-5.9	-8.8		-3.1	-1.1	-0.1	-4.4
Week 40	467	502.6	458.7	447.0	**429.4***	262.8	255.6	261.0	255.6	244.7
% change from control		7.6	-1.8	-4.3	-8.1		-2.7	-0.7	-2.7	-6.9
Week 52	486.9	**532.0***	486.3	470.7	451.5	275.8	265.6	272.7	268.8	**251.9***
% change from control		9.3	-0.1	-3.3	-7.3		-3.7	-1.1	-2.5	-8.7
Body weight gain (g)										
Week 13	185.9	205.5	175.6	168.5	**158****	87.6	79.8	81.8	86.5	**79***
% change from control		13.4	-5.5	-9.4	-15		-8.9	-6.6	-1.3	-9.8
Week 0 – 28	251.4	**285.2***	249.8	239.6	225.0	110.6	105.2	105.5	106.6	101.4
% change from control		13.4	-0.6	-4.7	-10.5		-4.9	-4.6	-3.6	-8.3
Week 40	282.3	313.5	275.9	266.9	**249.4***	122.6	114.7	117.5	114.6	106
% change from control		11.1	-2.3	-5.5	-11.7		-6.4	-4.2	-6.5	-13.5
Week 0 – 52	302.5	**343.8***	302.7	290.6	271.5	135.6	124.8	129.4	128.3	**113.3****
% change from control		13.7	0.1	-3.9	-10.2		-8.0	-4.6	-5.4	-16.4
Food consumption (g/animal/week)										
Week 1 – 28	155.7	**166.2***	157.0	149.0	148.3	119.9	115.4	114.9	115.5	111.3
Week 1 – 52	154.5	**163.9***	155.5	147.5	147.8	119.4	114.9	114.4	115.1	110.4*
Haematology (Week 52)										
Hb (g/dL)	16.1	16.2	16.1	16.0	**15.6****	15.6	15.4	15.2	**14.9*****	**14.7*****
MCH (pg)	17.7	17.5	17.7	17.4	**16.9****	19.0	18.7	**18.5***	18.8	**18.4****
MCHC (g/dL)	35.6	35.2	35.2	35.0	**34.5****	35.9	35.4	**35.0*****	**35.2****	**34.5*****
PT (s)	22.3	**21.3***	21.5	**21.1***	**20.0*****	21.5	21.9	21.8	21.4	21.5
LUC (10^9/L)	0.0	0.0	0.0	0.0	**0.1***	0.0	0.0	0.0	0.0	0.0
Blood chemistry (Week 52)										
ALP (IU/L)	59	52	54	**49***	50	27	28	24	**19****	**16*****

Ca (mmol/L)	2.64	2.62	2.63	2.66	2.63	2.69	**2.62****	2.69	**2.74***	**2.78*****
Cl (mmol/L)	105	**104***	**103*****	**102*****	**102*****	103	103	103	103	102
Inorganic P (mmol/L)	1.2	1.3	1.3	1.3	1.4**	0.9	0.9	0.8	0.9	1.0
Total protein (g/L)	68	69	69	70	69	72	71	73	75*	74*
Albumin(g/L)	44	45	45	**47***	46	53	51	52	54	54
Globulin (g/L)	24	24	23	23	23	19	20	20	**21***	20
A/G ratio	1.8	1.9	1.9	**2.0***	2.0	2.8	2.6	2.7	2.6	2.7
Total bilirubin (µmol/L)	1.7	1.9	1.8	1.7	1.5	2.8	2.4	2.4	2.7	**2.1****
γ-glutamyl transferase (IU/L)	2	2	2	2	**8*****	2	2	2	2	**6*****
Total chole-sterol (mmol/L)	2.3	2.3	2.4	2.7	**2.9***	2.0	1.8	2.3	**3.1*****	**3.0*****
Glucose (mmol/L)	6.3	6.1	**5.4****	5.7	5.9	6.1	5.5	**5.5***	**5.2*****	**4.8*****
Organ weights										
Terminal body weight (g)	476.9	**518.7***	473.4	458.8	439.2	267.3	256.3	262.7	258.7	**244.1***
Liver (g)	9.631	**11.225****	10.494	**10.771***	**12.275*****	6.215	5.961	6.409	**7.258****	**7.705*****
Relative weight (Ratio %)	2.014	2.157	**2.218***	**2.350*****	**2.816*****	2.328	2.335	2.439	**2.826*****	**3.150*****
Macroscopic findings										
Liver										
Large	0/19	0/19	0/19	1/20	**6***/20	0/20	0/20	1/19	0/19	2/20
Microscopic findings										
No. examined	19	19	19	20	20	20	20	19	19	20
Liver										
Hepatocellular eosinophilia/hypertrophy	0	0	0	**15*****	**20*****	0	0	0	**17*****	**15*****
Hepatocyte vacuolation	1	3	1	3	6	0	2	3	4	2
Thyroid										
Follicular cell hypertrophy	1	0	1	**9****	**18*****	0	0	0	**9*****	**15*****

* p<0.05; **p<0.01; ***p<0.001

[#] No. of animals that died prior to termination / No. of animals in group.

Table 46: Key results for the combined chronic toxicity/carcinogenicity study in rats – Carcinogenicity cohort

	Males					**Females**				
Diet concentration (ppm)	**0**	**400**	**2000**	**7000**	**15000**	**0**	**400**	**2000**	**7000**	**15000**
S-2200 intake (mg/kg bw/day)										
	0	21.0	105.1	375.6	804.3	0	26.7	135.2	475.0	1016.2
Mortality[#]										
	18/50	8/50	18/50	9/50	14/50	17/50	15/50	14/50	10/50	9/50

Survival %										
	64	84	64	82	72	66	70	72	80	82
Body weight (g)										
Week 0	188.3	187.6	188.7	186.4	188.9	144.0	141.7	143.4	143.8	144.4
Week 104	611.5	613.4	570.6	584.5	**538.5** ***	363.6	353.8	**323.2****	**323.2****	**292.5** ***
Body weight gain (g)										
Week 0 – 52	327.5	329.8	331.7	314.5	**291.8** ***	139.9	143.5	130.7	**120.9** ***	**110.8** ***
Week 0 – 104	424.0	426.5	384.4	396.7	**349.2** ***	220.0	214.5	**180.1****	**179.9****	**147.9** ***
Food consumption (g/animal/week)										
Week 1 – 52[α]	152.2	151.2	153.6	150.2	**144.6***	113.5	114.2	113.1	110.6	107.2
Week 1 – 104[α]	148.4	147.4	149.4	147.2	141.9	113.6	114.1	112.8	111.6	108.1
Haematology (Week 104)										
WBC (10^9/L)	5.9	**4.7****	**4.8***	5.3	5.1	3.4	3.5	4.1	3.5	4.3
L (10^9/L)	3.9	3.1*	3.3	3.3	3.5	2.1	2.0	2.1	2.0	2.0
E (10^9/L)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	**0.4***
Organ weights										
Terminal body weight (g)	595.3	598.6	561.4	569.9	**531.1****	348.6	339.3	**309.8****	**309.7****	**278.8** ***
Liver (g)	11.512	11.873	11.171	13.104	11.848	6.989	7.262	7.340	7.226	7.748
Relative weight (Ratio %)	2.051	2.029	2.042	2.215	**2.334***	2.153	2.038	**2.472***	**2.446***	**2.753** ***
Macroscopic findings										
Liver (No. examined per group: 50)										
Large	2	4	4	7	9	0	2	2	2	3
Non-neoplastic Microscopic findings										
Liver										
No. examined	32	42	32	41	36	33	35	36	40	41
Hepatocellular eosinophilia/ hypertrophy	6	10	12	**28*****	**35*****	15	18	**30****	**36*****	**37*****
Hepatocyte vacuolation	22	37	26	35	**33***	15	10	18	**29***	**34****
Bile duct hyperplasia	2	5	7	4	4	10	17	18	**26****	18
Thyroid										
No. examined	32	42	32	40	36	33	35	36	40	41
Follicular cell hypertrophy	0	2	0	0	**10****	0	0	0	0	3
Kidney										
No. examined	32	42	32	41	36	33	35	36	40	41
Papillary mineralisation	7	8	7	10	13	17	13	25	**30***	26
Pelvic mineralisation	22	30	19	30	26	30	29	34	36	36
corticomedullary mineralisation	0	1	0	0	0	6	7	**14***	**16***	14
Ovary										
No. examined	-	-	-	-	-	50	50	50	50	50
Sex-cord stromal	-	-	-	-	-	3	8	5	6	5

hyperplasia										
Neoplastic Microscopic findings										
Benign ovary sex-cord stromal tumours	-	-	-	-	-	2/50 exceed HCD	0/50	1/50	4/50 exceed HCD	6/50 exceed HCD
Benign mammary fibroadenomas	-	-	-	-	-	12/48	2/20	5/21	4/18	5/50
Benign interstitial cell adenomas in testis	0/50	0/13	0/22	2/15	3/50 within HCD	-	-	-	-	-
Pituitary adenomas + carcinomas	9/50	4/12	16/28	8/18	11/50	28/50	20/35	18/31	19/29	**18*/49**

* p<0.05; **p<0.01; ***p<0.001
# No. of animals that died prior to termination / No. of animals in group.

CONCLUSION:

Due to the magnitudes of the decreased body weight and body weight gain (at 15000 ppm) and toxicological alterations in the liver including increased liver weights in combination with a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (both at ≥ 7000 ppm) and/or increased blood biochemistry parameters (total cholesterol and gamma glutamyltransferase in males offered 15000 ppm), the No Observed Adverse Effect Level (NOAEL) for males was considered to be 2000 ppm (105.1 mg/kg bw/day).

For females, body weight and body weight gain was significantly decreased at ≥ 2000 ppm following 104 weeks of treatment. Toxicological alterations in the liver in females included increased liver weights (at ≥ 2000 ppm), a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (at ≥ 2000 ppm and at ≥ 7000 ppm, respectively) and/or increased blood biochemistry parameters (increased total cholesterol and increased gamma glutamyltransferase at ≥ 7000 ppm and at ≥ 15000 ppm, respectively). Therefore, the NOAEL for females was considered to be 400 ppm (26.7 mg/kg bw/day) for this study following 104 weeks of treatment.

Regarding the carcinogenic potential of S-2200 TG, no increase of neoplastic findings exceeding the historical control range was observed in any organ of treated animals, with exception of benign sex-cord stromal tumours in the ovary. Four and six cases occurred in female rats of the carcinogenicity cohort dosed with 7000 ppm and 15000 ppm, respectively, at dose levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded. S-2200 TG is not genotoxic. The incidence of only one benign tumour type was increased. Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. The increased incidence of sex-cord stromal tumour in the ovary at 7000 ppm and 15000 ppm

exceeded the Covance, Harrogate historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that also the concurrent control animals (incidence 2/50 = 4%) exceeded the historical control range for sex-cord stromal tumours.

Since there is no interaction of the test article with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays, no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of S-2200 TG or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay, the number of tumours in any tissue was not increased by exposure to S-2200 TG. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

Overall, according to Regulation (EC) No. 1272/2008 and in consideration of the *Guidance on the Application of CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0 April 2012*, no classification and labelling as carcinogenic substance is proposed for the active substance mandestrobin.

Mice

Reference:	S-2200 Technical Grade: 78 Week Oral (Dietary) Administration Carcinogenicity Study in the Mouse
Author(s), year:	Beck, W.; 2012c
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0073
Guideline(s):	OECD 451 (1981), Reg. (EC) No. 440/2008, B32 (2008), EPA OPPTS 870.4200 (1998), Japanese MAFF 12 Nohsan 8147 (2-1-15) (2001)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	None
Validity:	Yes

MATERIAL AND METHODS:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Stability of test compound:	Expiry date: 21 November 2011 (after completion of treatment)
Vehicle:	None. Test material was mixed directly into diet.
Test animals:	
Species:	Mouse
Strain:	Crl:CD1(ICR)
Age:	6 to 7 weeks old at the start of treatment
Weight at dosing:	29.4 to 40.1 g for males and 20.5 to 30.6 g for females
Source:	Charles River Laboratories, Margate, UK
Diet:	Finely ground SQC Rat and Mouse Maintenance Diet No 1 (Special Diets Services Ltd, Witham, UK) *ad libitum*

The purpose of this study was to assess the carcinogenic potential of mandestrobin (S-2200 TG) after dietary administration to mice for 52 weeks (satellite groups; 12 animals per sex and dose group) and 78 weeks (main groups; 51 animals per sex and dose group).

Animal assignment and treatment:

63 animals per sex were assigned to each treatment group using a total randomisation procedure. 12 animals per sex (i.e. satellite group) in each treatment group were used for interim sacrifice and examination at 52 weeks. Following the first full weighing (Day -7), group mean body weights and standard deviations were calculated and inspected to ensure there were no unacceptable differences between the groups. The mice received S-2200 TG in the diet at concentrations of 0 (control), 700, 2000 and 7000 ppm.

Diet preparation and analysis:

Formulations were prepared weekly. The test article was formulated as a diet mix in SQC Rat and Mouse Maintenance Diet No 1 (ground fine).

Formulations were analysed for their test article content and homogeneity using High Performance Liquid Chromatography with Ultra Violet detection (HPLC-UV), based on a method supplied by the Sponsor, and was validated in Covance Study Number 8201819.

The test article was demonstrated to be stable in diet at 100 and 20000 ppm when stored for 15 days at ambient temperature (Covance Study Number 8201819).

Table 47: Group mean compound intakes (mg/kg bw/d) following 52 (satellite group) and 78 (main group) weeks of treatment

Group	**Dose level (ppm)**	**Mean compound consumption (mg/kg bw/d)**			
		Satellite group		**Main group**	
		Male	**Female**	**Male**	**Female**
1 (control)	0	-	-	-	-
2 (low)	700	88.4	104.0	82.5	99.2
3 (intermediate)	2000	255.0	325.0	238.8	280.3
4 (high)	7000	883.3	1045.1	823.9	994.0

Clinical observations:

All animals were observed at the beginning and the end of the working day to ensure they were in good health. Any animal which showed marked signs of ill health was isolated. All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a detailed physical examination at weekly intervals. An individual record was maintained of the clinical condition of each animal.

Body weight, food consumption and compound consumption:

Individual body weights were recorded on Day -7, once weekly from Day 1 (before dosing) to Week 16, once every 4 weeks thereafter and on the day of (prior to) necropsy. In addition to occasions stated in the protocol, main group body weights were also recorded in Weeks 77 and 78.

The amount of food consumed by each cage of animals was determined weekly from Week -1 to Week 16 and on one week in every four thereafter. In addition to occasions stated in the protocol, main group food consumption was also recorded in Weeks 77 and 78. Consumption was calculated as g/animal/week.

Compound consumption was calculated once weekly from Week 1 to Week 16 and on one week in every four thereafter, and in addition also in Weeks 77 and 78. Weekly compound consumption was calculated as mg/kg/day and also as an average consumption over the entire treatment period (mg/kg/day).

Haematology:

Blood samples (nominally 0.5 mL into EDTA anticoagulant) were withdrawn by orbital sinus puncture from satellite group animals in Week 52 and from main group animals in Week 78. Blood samples were also taken from decedents, where possible.

Samples were analysed for total and differential white cell count. A blood film was routinely prepared but only examined where an assessment of cell morphology was useful to support or clarify abnormalities identified by the automated analyser.

Sacrifice and pathology:

All animals, including the decedents, were subjected to necropsy.

Where possible, the scheduled necropsies were performed in replicate order. Each animal was given an intraperitoneal overdose of sodium pentobarbitone. Once a suitable deep plane of anaesthesia was established, the animal was exsanguinated by the severing of major blood vessels. A full macroscopic examination was performed under the general supervision of a pathologist and all lesions were recorded.

The following tissues from all animals were preserved in the appropriate fixative.

Table 48: Tissues preserved in appropriate fixative*; W = weighed; E = processed and examined microscopically

Adrenals	W	E	Oesophagus		E
Animal identification			Optic nerves		E
Aorta		E	Ovaries	W	E
Brain	W	E	Pancreas		E
Caecum		E	Pituitary		E
Colon		E	Prostate		E
Duodenum		E	Rectum		E
Eyes (a)		E	Salivary glands – mandibular, sublingual, parotid		E
Femur with bone marrow and stifle joint		E	Sciatic nerves		E

Gall bladder		E	Seminal vesicles		E
Gross lesions		E	Skin and subcutaneous tissue		E
Harderian glands		E	Spinal cord – cervical		E
Head			Spinal cord – lumbar		E
Heart	W	E	Spinal cord thoracic		E
Ileum		E	Spleen	W	E
Jejunum		E	Sternum with bone marrow		E
Kidney	W	E	Stomach		E
Lacrimal glands			Testes and epididymides (b)	W	E
Larynx		E	Thymus		E
Liver	W	E	Thyroids and parathyroids		E
Lungs with mainstem bronchi and bronchioles		E	Tissue masses		E
Mammary		E	Tongue		
Mandibular lymph nodes		E	Trachea		E
Mesenteric lymph nodes		E	Trachea bifurcation		
Muscle (quadriceps)		E	Urinary bladder		E
Nares		E	Uterus including cervix #	W	E
Nasal cavity		E	Vagina		E
Nasopharynx		E			

* Fixative = neutral-buffered 10% formalin except where indicated by:
a – Davidson's fluid
b – Bouin's fixative
Bone designated for histopathological examination was decalcified using Kristenson's fluid.

Organ Weights:

Animals were weighed before necropsy. The organs denoted by "W" in the table (tissue list) above from all interim group animals, excluding decedents, and generally from the first 10 scheduled main group animals/group/sex were dissected free from fat and other contiguous tissue and weighed before fixation. Left and right organs were weighed together.

It must be mentioned that the thyroid weight was not recorded as detailed in the updated OECD guideline No. 453 from 07 September 2009. However, pathological examination was conducted. Therefore, this omission is considered not to have affected the outcome or integrity of the study.

Histopathology:

The following tissues were embedded in paraffin wax BP (block stage), sectioned at a nominal 5 µm, stained with haematoxylin and eosin (slide stage) and examined microscopically by the Study Pathologist:

Satellite Group:

Group 1 (control) and Group 4 (high dose): Liver, gross lesions and tissue masses

Decedents (all dose groups): All tissues denoted by "E" in the table (tissue list) above

Main group:

Group 1, Group 4 and all decedents: All tissues denoted by "E" in the table (tissue list) above

Group 2 (low dose) and Group 3 (intermediate dose): Liver, gross lesions and tissue masses

Csaba stain was used on selected tissues from animals 33, 442, 459 and 480 to rule in or rule out mast cell tumours.

Statistics:

Analysis of survival data:
Male and female data were analysed separately. Survival probability functions were estimated by the Kaplan Meier technique. Survival curves were compared to the start of the terminal kill phase (during Week 79). Permutational tests for both an increasing and a decreasing dose response in mortality were performed across all groups using the dose levels as weighting coefficients, in accordance with the IARC annex. One-directional pairwise tests of the treated groups against the control group were also performed.

Analysis of in-life and organ weight parameters:
The control group was taken as the baseline group with which the treated groups were compared. Absolute body weights, body weight gains, necropsy body weights, organ weights, organ to necropsy body weight ratios, food consumption over periods and selected haematology variables (white blood cell counts, neutrophils and lymphocytes) were analysed using one-way analysis of variance (ANOVA), separately for each sex. Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was used to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was reported only where none of the pairwise comparisons was significant.
Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed either using the same methods after applying a log-transformation or using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.
Selected haematology variables (monocytes, eosinophils, basophils and large unstained cells) were analysed using the non-parametric methods described above.

Analysis of tumour (neoplastic) data:
All tumour data for all tissues examined were supplied for analysis. Male and female data were analysed separately. The numbers of tumour bearing animals were analysed for tumour types found in at least three animals of the given sex. Tumours of similar histogenic origin were merged, as requested by the Pathologist. Permutational tests for both an increasing and a decreasing dose response were performed across the groups using the dose levels as weighting coefficients, in accordance with the IARC annex. One-directional pairwise tests of the treated groups against the control group were also performed.
Non-fatal tumours were analysed using fixed intervals of 1 to 52 weeks, 53 to 78 weeks, the interim kill phase and the terminal kill phase. The fatal and non-fatal results were combined in accordance with the IARC annex. For each macroscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher’s Exact test.

Analysis of macroscopic/microscopic (non-neoplastic) findings:
All macroscopic and microscopic finding data for all tissues examined were supplied for analysis. Male and female data were analysed separately. All tests were performed with a two-sided risk.

At the request of the Study Director, four separate analyses were performed; including all animals, decedents only, terminal kill animals only and interim kill animals only. For each macroscopic or microscopic finding, comparisons were made between the incidence in each of the treated groups and that in the control group, using Fisher's Exact test.

FINDINGS:

Mortality and clinical observations:

There was no effect of treatment on survival. Mortalities are detailed in the summary table below (Table. 49). Survival was acceptable for deriving a conclusion with regard to carcinogenicity. Of the 119 decedents, there were 16 animals where a cause of demise was not determined. In addition to the findings associated with terminal events, the range of macroscopic and microscopic findings in decedents was generally similar to those in animals surviving to terminal kill.

The general ranges of clinical signs observed were considered typical of laboratory maintained mice and were not adversely affected by treatment. Clinical signs seen in moribund animals generally included hunched posture, sluggishness, pale appearance, laboured/rapid respiration, swollen abdomen and/or thin appearance. The incidences and causes of morbidity and mortality in controls and treated animals were generally similar and consistent with the usual pattern of causes of demise in mice of this strain.

Body weight, body weight gain and food consumption:

In the satellite groups, no statistically significant effects on bodyweight or body weight gain were seen in the pairwise comparisons. Satellite group females offered 7000 ppm showed approximately 10% less body weight than control between Week 40 to 52, while females in the main study using more animals did not show these findings over this designated period. Therefore these changes were considered to be not adverse.

In the main study, statistically significant differences from the control for absolute body weight were observed at some of the measurement occasions for males offered 2000 ppm (Week 13) or 7000 ppm (Weeks 5, 9, 20 and 36), which resulted in a significant reduction in body weight gain from Start to Week 28 (17% less) for the high dose males. From Weeks 52 to 78, a statistically significant body weight gain increase was observed for males offered 2000 or 7000 ppm mainly as a result of mean body weight loss in the contemporaneous control group over the same period.

There were no statistically significant effects on absolute body weight of main study females. The overall growth rate of females offered 700 ppm was similar to control. The overall mean body weight gain of females offered 2000 ppm and 7000 ppm was 8% more and 8% less than that of the control group, respectively.

For both the satellite group and the main group, there were no statistically significant differences in food consumption when compared to control.

Haematology:

In samples collected at the Week 52 (interim) or 78 (main study) investigations, there were no statistically significant treatment related differences in the haematological composition of the blood between control and treated animals.

A small number of control and treated animals killed during the treatment period or surviving to termination had elevated white blood cell counts generally due to increased neutrophils and/or lymphocytes, which generally correlated microscopically with the presence of haemolymphoreticular tumour.

Sacrifice and pathology

Organ weights:

Organ weight increases were noted in the liver of treated males and females (see Table 49). Increases of relative liver weights were statistically significant in the high dose males of the interim study as well as from the main study and in high dose females from the interim study. In the absence of histopathological findings in the liver, these increases were considered to be an adaptive change related to drug metabolism and not an adverse toxic effect.

The mode of action for the liver weight increase is considered to be due to liver enzyme induction, via activation of the constitutive androstane receptor by S-2200 TG, as evidenced by mode of action investigations (see mechanistic studies in section 4.12.1.3). Because a mode of action is ascribable, the increase in liver weights is considered non-adverse, particularly in the absence of any other biochemical or histological indicators of an adverse effect on the liver.

All other changes in absolute or relative organ weights were not statistically significant when compared with controls by pair-wise analysis and were furthermore considered not to be relevant as they were either small in magnitude, not dose-dependent, inconsistent between the sexes, spontaneous background changes due to normal inter-animal variability and/or lacked a histopathological correlate.

Macroscopic findings:

Most tissues were macroscopically unremarkable, and the findings seen were generally consistent with the usual pattern of findings in mice of this strain and age. There were no macroscopic findings that could be discerned as treatment-related.

Microscopic findings:

Non-neoplastic microscopic findings in control and treated animals were generally consistent with the usual pattern of findings in mice of this strain and age. There were no non-neoplastic findings that could be related to treatment with the test article.

Neoplastic microscopic findings in control and treated animals were generally consistent with the usual pattern of neoplasms in mice of this strain and age. There was no statistically significant increase or decrease in tumour incidence. There were no tumours suggestive of test article carcinogenicity.

Table 49: Key results for the carcinogenicity study in mice

	Males				**Females**			
Diet concentration (ppm)	**0**	**700**	**2000**	**7000**	**0**	**700**	**2000**	**7000**

Mortality								
Mortality[#] (satellite groups)	2/12	0/12	1/12	2/12	2/12	0/12	1/12	0/12
Survival % (satellite groups)	83	100	92	83	83	100	92	100
Mortality[#] (main groups)	13/51	12/51	9/51	13/51	18/51	16/51	16/51	14/51
Survival % (main groups)	75	76	82	75	65	69	69	73
Body weight (g)								
Week 0 (satellite groups)	33.7	34.7	33.7	33.4	25.0	24.5	25.1	24.2
Week 52 (satellite groups)	52.0	50.8	49.2	49.3	43.4	42.3	44.1	37.3
Week 0 (main groups)	34.3	34.6	34.5	34.2	24.4	24.5	24.4	24.6
Week 78 (main groups)	51.2	51.3	51.8	50.3	43.1	43.6	44.7	41.6
Body weight gain (g)								
Week 0-28 (satellite groups)	12.4	11.6	12.1	12.1	10.9	10.0	11.3	9.8
Week 28-52 (satellite groups)	6.1	4.5	3.4	4.2	7.4	7.8	8.1	3.3
Week 0-28 (main groups)	13.9	13.9	12.1	**11.6***	10.6	11.4	11.0	11.2
Week 28-52 (main groups)	4.1	5.4	4.7	5.0	7.3	6.6	7.9	6.1
Week 52-78 (main groups)	-1.4	-1.5	**0.1***	**0.1***	0.8	0.8	-0.2	-0.3
Organ weights								
Liver, wk 52 (g)	2.34	2.47	2.43	2.59	1.75	1.80	1.93	1.97
% difference from control		5.6	3.8	10.7		2.9	10.3	12.6
Liver, wk 52 (% bw)	4.42	4.67	4.76	**5.10****	4.07	4.19	4.41	**5.10****
% difference from control		5.7	7.7	15.4		2.9	8.4	25.3
Liver, wk 78 (g)	2.40	2.55	2.54	2.76	1.93	2.20	2.32	2.09
% difference from control		6.3	5.8	15.0		14.0	20.2	8.3
Liver, wk 78 (% bw)	4.59	4.85	4.81	**5.24****	4.42	4.65	4.96	4.90
% difference from control		5.7	4.8	14.2		5.2	12.2	10.9

* $p < 0.05$ in comparison to controls, ** $p<0.01$ in comparison to controls.

[#] No. of animals that died prior to termination / No. of animals in group.

CONCLUSION:

Treatment with S-2200 Technical Grade was well-tolerated. In the absence of adverse effects, the NOAEL for this study was considered to be 7000 ppm (823.9 mg/kg bw/day for males and 994.0 mg/kg bw/day for females), the top dose tested, following 78 weeks of treatment. There were no effects on survival/mortality or on the incidence or morphology of tumours to indicate any oncogenic potential.

4.10.1.2 Carcinogenicity: inhalation

No data available.

4.10.1.3 Carcinogenicity: dermal

No data available.

4.10.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.10.3 Other relevant information

Several *in vivo* and *in vitro* mechanistic studies were performed, and two detailed position papers were submitted dealing with (i) liver and thyroid effects and (ii) ovary issues. These studies and position papers are reported and evaluated in section *4.12.1.3 Specific investigations: other studies*.

4.10.4 Summary and discussion of carcinogenicity

Groups of 70 male and 70 female Wistar rats were offered mandestrobin in the diet at concentrations of 0 (control), 400, 2000, 7000, 15000 ppm. After 52 weeks, satellite groups of 20 males and 20 females were used for interim sacrifice and the remaining survivors sacrificed after 104 weeks of treatment.

Due to the magnitudes of the decreased body weight and body weight gain (at 15000 ppm) and toxicological alterations in the liver including increased liver weights in combination with a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (both at ≥ 7000 ppm) and increased blood biochemistry parameters (total cholesterol and gamma-glutamyltransferase in males offered 15000 ppm), the NOAEL for males was considered to be 2000 ppm (105.1 mg/kg bw/day).

For females, body weight and body weight gain was significantly decreased at ≥ 2000 ppm following 104 weeks of treatment. Toxicological alterations in the liver in females included increased liver weights (at ≥ 2000 ppm), a higher degree of hepatocellular hypertrophy and hepatocyte vacuolation (at ≥ 2000 ppm and at ≥ 7000 ppm, respectively) and increased blood biochemistry parameters (increased total cholesterol and increased gamma glutamyltransferase at ≥ 7000 ppm and at ≥ 15000 ppm, respectively). Therefore, the NOAEL for females was considered to be 400 ppm (26.7 mg/kg bw/day) for this study following 104 weeks of treatment.

Regarding the carcinogenic potential of mandestrobin, no increase of neoplastic findings exceeding the historical control range was observed in any organ of treated animals, with exception of benign sex-cord stromal tumours in the ovary. Four and six cases occurred in female rats of the carcinogenicity cohort dosed with 7000 ppm and 15000 ppm, respectively, at dose

levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded. Mandestrobin is not genotoxic. The incidence of only one benign tumour type was increased. Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. The increased incidence of sex-cord stromal tumour in the ovary at 7000 ppm and 15000 ppm exceeded the Covance, Harrogate historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that also the concurrent control animals (incidence 2/50 = 4%) exceeded the historical control range for sex-cord stromal tumours.

Since there is no interaction of the test article with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays (see section *4.12.1.3 Specific investigations: other studies*), no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of mandestrobin or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay, the number of tumours in any tissue was not increased by exposure to mandestrobin. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

Overall, according to Regulation (EC) No. 1272/2008 and in consideration of the *Guidance on the Application of CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0 April 2012*, no classification and labelling as carcinogenic substance is proposed for the active substance mandestrobin.

Groups of 51 male and 51 female CD-1 mice were given mandestrobin in the diet at concentrations of 0 (controls), 700, 2000, and 7000 ppm for 78 weeks. Satellite groups of 12 mice per sex per dose were reared up to 52 weeks for interim sacrifice.

Treatment with mandestrobin was well-tolerated. In the absence of adverse effects, the NOAEL for this study was considered to be 7000 ppm (823.9 mg/kg bw/day for males and 994.0 mg/kg bw/day for females), the top dose tested, following 78 weeks of treatment. There were no effects on survival/mortality or on the incidence or morphology of tumours to indicate any oncogenic potential.

4.10.5 Comparison with criteria

By default, carcinogenic effects in experimental animals are considered relevant to humans and are considered for classification as carcinogens (*Guidance on the Application of CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labeling and packaging (CLP) of substances and mixtures, 2009*). Only if there is sufficient evidence showing non-relevance of a certain type of tumors to humans, this tumor type should be excluded for classification.

Mandestrobin is not genotoxic. The incidence of only one benign tumour type was increased. Four and six cases of benign sex-cord stromal tumours in the ovary occurred in female rats of the carcinogenicity cohort dosed with 7000 ppm and 15000 ppm, respectively, at dose levels where body weight gain was reduced by more than 20%, indicating that the maximum tolerated dose was exceeded.
Sex-cord stromal hyperplasia does occur in aged Wistar rats, and the animals used in this study

appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were within historical controls for all groups, and there was no statistically significant difference between groups for hyperplasia or tumours. Higher survival rates in animals offered 7000 and 15000 ppm may have contributed to the higher numbers of tumours. The increased incidence of sex-cord stromal tumour in the ovary at 7000 ppm and 15000 ppm exceeded the Covance, Harrogate historical control range (0-3.1%) for this strain of rat. However, it must be highlighted that also the concurrent control animals (incidence 2/50 = 4%) exceeded the historical control range for sex-cord stromal tumours.

Since there is no interaction of mandestrobin with the oestrogen receptor and steroidogenesis as evidenced by *in vitro* assays (see section *4.12.1.3 Specific investigations: other studies*), no direct ovarian toxicity and no reproductive abnormalities, a mode of action via endocrine imbalance is considered to be unlikely. Furthermore, there was no accumulation or persistence of mandestrobin or its metabolites in the ovary. In the corresponding mouse carcinogenicity bioassay, the number of tumours in any tissue was not increased by exposure to mandestrobin. Thus, based on this evidence, the sex-cord stromal lesions are considered unlikely to be toxicologically relevant.

Overall, according to Regulation (EC) No. 1272/2008 and in consideration of the *Guidance on the Application of CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0 April 2012*, no classification and labelling as carcinogenic substance is proposed for the active substance mandestrobin.

4.10.6 Conclusions on classification and labelling

No classification and labelling is proposed for mandestrobin regarding carcinogenicity.

4.11 Toxicity for reproduction

Table 50: Summary table of relevant reproductive toxicity studies

Method	Dose levels / NOAEL / Effects at LOAEL	Remarks	Reference
Dose range-finding study for Two-generation reproduction toxicity study (rat) (no appropriate guideline, pilot study)	0, 5000, 10000, 20000 ppm equivalent to: 0, 244 – 782, 499 – 1429, 1033 – 2441 mg/kg bw/d (rounded) <u>Parental NOAEL</u>: *Females:* 317 mg/kg bw/d (5000 ppm) *Males:* - (lowest dose tested was a LOAEL) Females: - Decreased bw, bw gain and food consumption - Liver weight increase, brown pigment in bile duct/perilobular hepatocytes, inflammatory cell infiltration in periductular region - Decreased vacuolation in the interstitial gland in the ovary - Decreased uterus weight and atrophy of the uterus Males: - Liver weight increase greater than 20% <u>Offspring NOAEL</u>:	Wistar, BrlHan: WIST@Jcl (GALAS) rats Purity: 93.4%	Hoshino, N.; 2010

	317 mg/kg bw/d (5000 ppm) - Suppressed body weight and body weight gain - Reduced spleen weight Reproduction NOAEL: 1229 mg/kg bw/d (20000 ppm) No effects at highest dose tested		
Two-generation reproduction toxicity study (rat) (OECD 416)	0, 1000, 3000, 10000 ppm equivalent to: 0, 43 – 163, 132 - 511, 452 – 1688 mg/kg bw/d (rounded) Parental **LOAEL**: 60.19 mg/kg bw/d (1000 ppm) *Liver:* Increased liver weight and diffuse hepatocellular hypertrophy Offspring NOAEL: 56 mg/kg bw/d (1000 ppm) Lower spleen weights at weaning (males) Reproduction NOAEL: 559 mg/kg bw/d (10000 ppm) No effects at highest dose tested	Wistar, BrlHan: WIST@Jcl (GALAS) rats Purity: 93.4%	Matsuura, I.; 2012
Developmental toxicity range finding study (rat) (no specific guideline, conducted to support OECD 414)	0, 250, 500, 1000 mg/kg bw/d Maternal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested Foetal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested; no teratogenic potential	Wistar, Crl:WI(Han) rat Purity: 93.4%	Rhodes, J.; 2009a
Developmental toxicity study (rat) (OECD 414)	0, 100, 300, 1000 mg/kg bw/d Maternal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested Foetal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested; no teratogenic potential	Wistar, Crl:WI(Han) rat Purity: 93.4%	Rhodes, J.; 2012a
Developmental toxicity range finding study (rabbit) (guideline not stated, range finding study)	0, 250, 500, 1000 mg/kg bw/d Maternal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested Foetal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested; no teratogenic potential	New Zealand White (Hsd:IfNZW) rabbit Purity: 93.4%	Rhodes, J.; 2009b
Developmental toxicity	0, 100, 300, 1000 mg/kg bw/d	New Zealand	Rhodes, J.;

study (rabbit) (OECD 414)	 Maternal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested Foetal NOAEL: 1000 mg/kg bw/d No treatment-related effects at highest dose tested; no teratogenic potential	White (Hsd:IfNZW) rabbit Purity: 93.4%	2012b

4.11.1 Effects on fertility

4.11.1.1 Non-human information

Reference:	Dose Range-Finding Study for Two-Generation Reproduction Study of S-2200 TG in Rats
Author(s), year:	Hoshino, N.; 2010
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0018
Guideline(s):	None
GLP:	No
Deviations:	One female of the low dose group was judged as non-copulation by vaginal smear examination during the mating period; however this female was proved to be pregnant. Consequently, several parameters during gestation period were not obtained (chemical intake, bw, food consumption). Data of lactation period was normally collected; no effect on evaluation of study results.
Validity:	Yes; supporting study

MATERIAL AND METHODS:

Test Material: S-2200 Technical Grade (S-2200 TG)
Lot/Batch: ST-0811G
Purity: 93.4%

Test animals:
Species: Rat
Strain: Wistar, BrlHan: WIST@Jcl (GALAS)
Age: 8 weeks old at start of administration
Weight at start: Males 205 – 228 g, females 149 – 168 g
Source: CLEA Japan, Inc.
Diet: CRF-1 (Oriental Yeast Co., Ltd.) sterilized by gamma rays *ad libitum*

The purpose of this dose range-finding study was to obtain information for selecting dose levels for the definitive study aimed at assessing effects of mandestrobin (S-2200 TG) on the reproductive system, including mating, gestation, parturition and lactation of the parental animals by dietary administration from 4 weeks before mating to Day 21 of lactation in rats.

Animal assignment and treatment

Groups of 10 male and 10 female young adult rats were offered S-2200 TG in diet at dietary concentrations of 0 (control), 5000, 10000 or 20000 ppm. The dose levels were selected based on the results of a thirteen-week dietary toxicity study of S-2200 TG in rats (Study No. 0333/290, dose levels: 0, 800, 4000, 10000, and 20000 ppm). A dose level of 20000 ppm was selected as the highest dose for this study, at which some toxic signs occurred in the thirteen week study (decreased body weight and suppressed body weight gain in both sexes). The intermediate and low doses were set at 10000 and 5000 ppm, respectively, by decreasing geometrically based on a factor of 2.

Males were dosed daily for 4 weeks before mating and through the mating period until the day of necropsy. Females were dosed daily for 4 weeks before mating and through the mating period until weaning of F1 offspring (Day 21 of lactation).

Diet preparation and analysis:

The mixed diet with the test substance was prepared once within 14 days. For each dose, a prescribed amount of the test substance and the primary diet were weighed. Then, the test substance and the proper primary diet were ground and mixed with a compact mill, and further primary diet was added in a stepwise manner to achieve the prescribed concentrations of the test substance in diet. The mixed diet with the test substance was subdivided into portions for one week in plastic bags. The primary diet for the control group was also subdivided into portions in plastic bag in the same manner without any preparation.

The prepared diet was refrigerated and stored in a dark place until use. No analysis of concentration, stability or homogeneity in the diet was made.

Mean test substance intakes (mg/kg bw/d) are given in Table 51.

Table 51: Mean test substance intakes (mg/kg bw/d) – F0 animals only

Sex	**Doses** **Period of treatment**	**5000 ppm (Group 2)**	**10000 ppm (Group 3)**	**20000 ppm (Group 4)**
males	Before mating	317.9	636.0	1253.0
	After mating	244.1	498.5	1032.5
females	Before mating	334.6	667.1	1326.0
	Gestation	316.8	667.8	1229.0
	Lactation	781.7	1429.2	2441.0

Observation and examination of parental animals (F0)

Clinical observations:

The animals were observed by cage-side once daily and were observed by palpation once weekly.

Body weight:

Body weights were measured once a week from the first day of dosing to necropsy for males and to copulation for females. The mated females were weighed on Days 0 (day of copulation), 7, 14, and 20 of gestation and on Days 0 (day of parturition), 4, 7, 14, and 21 of lactation. Males and copulated females were weighed on the day of necropsy. Body weight gain was calculated on the

basis of the body weight on the first day of dosing during the pre-mating period, Day 0 of gestation during the gestation period, and Day 0 of lactation during the lactation period.

Food consumption:

Gross weight of the diet for each cage was measured once a week from the first day of dosing to 4 weeks after the start of dosing for males and females during the pre-mating period. For mated females, gross weight of the diet was individually measured on Days 0, 7, 14, and 20 of gestation and Days 0, 7, 14, and 21 of lactation. For mated males, gross weight of the diet was weighed in the same interval re-starting from the nearest measurement day to the day of necropsy. Daily food consumption of each animal was calculated by dividing the difference of the measured gross weights by the number of days between each measurement.

Reproductive function:

Males and females were cohabited day and night on one-to-one basis within the same group for up to 14 days (first mating). From the day after the start of mating, vaginal smears were collected every day in the morning, while observing for the presence of vaginal plugs, and the smears were examined microscopically to determine oestrous cycles and the presence of sperm. Copulation was confirmed by the presence of a vaginal plug or sperm in the vaginal smears, and the day on which the evidence of copulation was found was designated as day 0 of gestation. As a result of the first mating, all females of each group showed evidence of copulation by 14 days after the start of mating. Therefore, the test substance was judged to have no effect on the mating ability, and mating was terminated. Based on the above results, the following indices were calculated:

- Days until copulation: Number of days from the start of mating to the detection of copulation
- Number of oestrus stages without copulation
- Copulation index (%): (Number of animals with successful copulation/Number of paired animals) × 100
- Fertility index (%): (Number of pregnant animals/Number of animals with successful copulation) × 100

Observation of parturition and nursing:

For parturition, the dams were observed twice a day from Day 21 through Day 23 of gestation. The animals that delivered their litter completely by 4:00 p.m. were judged as dams giving birth on that day. For nursing, the dams were observed once a day for maternal behaviour, including lactation, nest building, and cannibalism, until Day 21 of lactation. At the necropsy on Day 21 of lactation, the uterus was removed and examined for the number of implantations. The following indices were calculated based on these results:

- Gestation length: Number of days from Day 0 of gestation to the day of parturition
- Gestation index (%): (Number of females with live offspring/Number of pregnant females) × 100
- Birth index (%): (Number of offspring born alive/Number of implantations) × 100

Necropsy:

Four days after delivery, males were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the thoracic and abdominal organs/tissues were examined macroscopically. Two males (#00109 and 00309) that displayed delayed copulation compared to other mated males were necropsied before Day 4 of lactation of the mating pair, because the no effects of test substance on gestation index or the number of live offspring at the delivery had already been concluded. The females were necropsied on Day 21 of lactation in the same manner as the males.

The thyroid, liver, testis, epididymis, prostate (ventral, lateral, and dorsal lobe), seminal vesicle (including coagulating gland), ovary, uterus, and mammary gland were fixed and preserved in a 10 vol% neutral phosphate-buffered formalin. The testes were fixed in Bouin's fluid and preserved in 10 vol% neutral phosphate-buffered formalin. Stomachs showing abnormal signs were also fixed and preserved in 10 vol% neutral phosphate-buffered formalin. The stomachs of 3 females in the control group was also fixed and preserved in the same manner as comparative samples.

Organ weights:

At necropsy, body weights and the following organs were weighed:

Thyroid, liver, testis, epididymis, prostate (ventral lobe), seminal vesicle (including coagulating gland), ovary, uterus (including cervical region).

Relative organ weights (body weight ratio) were calculated based on each body weight at necropsy. The thyroid was removed and weighed after fixation in 10 vol% neutral phosphate-buffered formalin. The bilateral organs were weighed individually and calculated as a sum.

Histopathology:

The liver and thyroid from all animals and the ovaries and uterus from all females were processed by the standard method to prepare haematoxylin- and eosin-stained (HE) sections for microscopic examination.

Observation and examination of offspring (F1)

Litter examinations:

The birthday was designated as postnatal Day 0.

On postnatal Day 0, the newborns were examined for the number of offspring (live or stillborn), sex, and presence of external anomalies. After that, the pups were observed daily for clinical signs and mortality until postnatal Day 21. On postnatal Day 4, litter size was reduced randomly to 8 pups (equal sex ratio, in principle). The litter with less than 8 pups was not subjected to adjustment. The offspring culled at the litter size adjustment were euthanized by inhalation of carbon dioxide and preserved in a 10 vol% neutral phosphate-buffered formalin. From the numbers of live offspring on postnatal Days 0, 4, 7, 14, and 21 (weaning day), the following indices were calculated:

- Live birth index (%) = viability index on Day 0 (%): (Number of live offspring born alive/Number of offspring born) × 100

- Viability index on Day 4 (%): (Number of offspring alive on Day 4/Number of offspring born alive) × 100
- Viability index on Day 7 (%): (Number of offspring alive on Day 7/Number of live offspring after culling) × 100
- Viability index on Day 14 (%): (Number of offspring alive on Day 14/Number of live offspring after culling) × 100
- Weaning index (%): (Number of live offspring at weaning/Number of live offspring after culling) × 100

Body weights:

The offspring were weighed individually on postnatal Days 0, 4, 7, 14, and 21. The body weight gain was calculated by each litter unit on the basis of the body weight at birth before the litter size adjustment, and by each offspring on the basis of the body weight on postnatal Day 4 thereafter.

Necropsy:

On postnatal Day 21, all F1 offspring were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the head, thoracic, and abdominal organs/tissues were examined macroscopically. The eyeball showing abnormal signs and brain, thymus, spleen, and uterus weighed on postnatal Day 21 were fixed and preserved in 10 vol% neutral phosphate-buffered formalin. The same organs of 3 females in the control group were also fixed and preserved in the same manner as the comparative samples. Dead pups (before the litter size adjustment) were examined for the presence of external anomalies and the whole bodies were preserved in 10 vol% neutral phosphate-buffered formalin. Cannibalized pups were stored in the same manner.

Organ weights:

At necropsy on postnatal Day 21, one male and one female were selected from each litter (in numerical order of the offspring number in each litter) and weighed for the following organs: Brain, thymus, spleen, and uterus.

Relative organ weights (body weight ratio) were calculated based on individual body weight at necropsy.

Statistics:

The data of offspring obtained before weaning were analysed on the basis of litter mean values.

The metrical data were analysed by multiple comparison tests for statistical significance. The homogeneity of variance was tested first by Bartlett's test. When the variance was homogeneous, the one-way analysis of variance was performed for statistical comparison. When it was heterogeneous, the Kruskal-Wallis test was used. When a significant inter-group difference was found, Dunnett's or the Dunnett-type multiple comparison test was used. For some of the data, the Kruskal-Wallis test was applied first, and when a significant inter-group difference was found, the Dunnett-type multiple comparison test was used. The numerical data were analysed by Fisher’s exact probability test. The significance level of 5% was set for all statistical analyses.

The statistical analyses were performed on the items listed below. The analyses were not performed on clinical signs and necropsy findings.

- Multiple comparison test: Body weight, body weight gain, food consumption, organ weights, number of implantations, number of offspring, number of live offspring
- Kruskal-Wallis test and Dunnett-type multiple comparison test: Days until copulation, number of oestrus stages without copulation, gestation length, birth index, live birth index, viability index on Days 4, 7, and 14, weaning index, incidence of offspring with external anomalies
- Fisher's exact probability test: Copulation index, fertility index, gestation index, sex ratio (male/female), dams with external abnormal offspring.

FINDINGS:

Effects on parental animals (F0)

Clinical signs:

No abnormal clinical signs were noted in parental males or females of any group, including the control group.

Body weight:

In males, statistically significant decreases in body weights and body weight gain were noted in the 20000 ppm group during the dosing period. No treatment-related changes were noted in the 5000 or 10000 ppm group.

In females, statistically significant decreases in body weight or body weight gain were noted in the 20000 ppm group from Day 28 of dosing through the gestation period to the lactation period. In females of the 10000 ppm group, statistically significant decreases in body weight or body weight gain were noted on Day 28 of dosing and Day 20 of gestation. No treatment-related changes were noted in the 5000 ppm group.

Food consumption:

In males, a statistically significant decrease in food consumption was noted in the high dose group (20000 ppm) on Day 7 of dosing; however, this change was considered to be not relevant, because it was transient and food consumption recovered by Day 14 of dosing. No treatment-related changes were noted in males at 5000 or 10000 ppm.

In females, statistically significant decreases in food consumption were noted in the high dose group (20000 ppm) on Day 7 of dosing and through the gestation period to the lactation period. In the mid dose group (10000 ppm), statistically significant decreases in food consumption were noted on Days 7 and 28 of dosing and during the lactation period. In the low dose group (5000 ppm), statistically significant decreases in food consumption were noted on Days 7 and 14 of dosing; however, these changes were considered to be not relevant, because they were transient and the food consumption recovered by Day 21 of dosing.

Reproductive performance, parturition and nursing:

All the mating pairs of each group copulated during the first oestrus stage after the start of mating, and no significant differences were noted in the number of days from the mating to the copulation. The copulation and fertility indices were 100.0% in all groups.

All dams in each group delivered normally between Days 21 and 23 of gestation and no significant differences were noted in the gestation length, number of implantations, birth index, or gestation index between the control and treated groups. In the observation of nursing, there were no abnormalities in maternal behaviour in dams of any group. The number of offspring born alive decreased in all treated groups, statistically significant in the high dose group.

Necropsy:

Dark brownish change in the liver was noted in one female in the mid dose group (10000 ppm), and in 10 males and 8 females in the high dose group (20000 ppm). Enlargement of the liver was noted in 5 males and 1 females at 10000 ppm, and in 10 males and 7 females at 20000 ppm. Enlargement of the thyroid was noted in 1, 1, and 4 males and in 0, 1, and 1 females in the 5000, 10000, and 20000 ppm groups, respectively.

Occasional other findings including abnormal lobation and reddish patch in the liver, mucosal reddish patch in the glandular stomach, and unilateral nodule in the epididymis were noted. However, these changes were considered to be incidental, because of the lack of dose-dependency and their pathological nature.

Organ weights:

Statistically significantly increased absolute and/or relative weights of the thyroid and liver were noted in males in all treated groups. In females, statistically significantly increased absolute and relative weights of the liver were noted, and decreased weights of the uterus were noted in the 10000 and 20000 ppm groups. In addition, decreased ovary weights were noted in the 20000 ppm group.

A statistically significant increase in relative weight of testis was noted in males in the 20000 ppm group; however, there was no significant difference in the absolute weight, as compared with the control group. Therefore, this change was considered attributable to the significantly lower body weight at necropsy, and was judged to be toxicologically insignificant.

Histopathology:

Liver: Brown pigment in the bile ducts was noted in 1 male and 5 females in the 10000 ppm group, and in 6 males and 9 females in the 20000 ppm group. Brown pigment deposition in the perilobular hepatocytes was noted in 2 females in the 10000 ppm group and in 3 males and 6 females in the 20000 ppm group. Focal periductular inflammatory cell infiltration was noted in 1 male and 3 females in the 10000 ppm group, and in 4 males and 8 females in the 20000 ppm group. Proliferation of the bile ducts was noted in 3 females in the 10000 ppm group and in 1 male and 5 females in the 20000 ppm group. Eosinophilic focus of the altered hepatocytes was noted in 1 male and 1 female in the 20000 ppm group. Centrilobular hypertrophy of the hepatocytes was noted in 4, 10, and 10 males and 6, 10, and 10 females in the 5000, 10000, and 20000 ppm groups, respectively.

The finding of focal necrosis of hepatocytes noted in 2 females in the 10000 ppm group was considered to be not relevant due to the lack of dose-response.

Thyroid: Diffuse hypertrophy of the follicular cells was noted in 2 males in the 10000 ppm group and in 5 males in the 20000 ppm group. Among 8 animals with enlarged thyroid at necropsy, diffuse hypertrophy of the follicular cells was noted in 2 males in the 20000 ppm group. In the other 6 animals, vacuolation of the follicular cells was noted. It has been reported that the vacuolation of thyroid follicular cells is noted as a spontaneous lesion in BrlHan:WIST@Jcl (GALAS) rats (Shimoi, A. et al., 2001, J Tox Pathol, 2001; 14:253-7). In the present study, this change was also noted in 1 female in the control group. Therefore, the vacuolation of the follicular cells was considered to be incidental.

Ovary: Decrease of vacuolation in the interstitial gland was noted in 1 female in the 10000 ppm group and in 7 females in the 20000 ppm group.

Uterus: Atrophy was noted in 2 females in the 10000 ppm group and in 10 females in the 20000 ppm group.

Effects on offspring (F1)

Litter examinations:

A statistically significant decrease in the viability index on Day 0 was noted in the 20000 ppm group. Furthermore, a decrease of weaning index (not statistically significant) was noted in the 20000 ppm group, and this change was induced by the death of 4 F1 animals after the culling in one dam.

A statistically significant decrease in the number of live offspring at birth (and as a consequence also in the number of live offspring on Day 4 before culling) was noted in the 20000 ppm group. However, the number of live offspring at birth in the control group was above the background range of the test facility, and that in the 20000 ppm group was slightly below the background range. Accordingly, it was considered that the decrease in the number of live offspring at birth in the high dose group was an incidental change attributable to the increased number of live offspring at birth in the control group, and was judged to be toxicologically insignificant.

A statistically significant decrease in the total number of male pups at birth (and in consequence, in the number of live males at birth and in the number live males on Day 4 before culling) was observed at 5000 ppm and at 10000 ppm. However, these changes were not considered to be treatment related because of the lack of dose-dependency.

External examination and clinical signs:

There were no external anomalies in the offspring of any group.

No abnormal clinical signs were noted in males or females or F1 pups of any group, including the control group.

Body weights:

Body weights of offspring were statistically significantly decreased in the mid dose group (10000 ppm) on postnatal Day 21 in both sexes and in the high dose group (20000 ppm) from postnatal

Days 7 and 4 onwards in males and females, respectively. Body weight gain was statistically significantly decreased in the mid dose group (10000 ppm) from postnatal Days 7 and 14 onwards in males and females, respectively, and in the high dose group (20000 ppm) in both sexes at every measurement (starting before culling at postnatal Day 4 up to weaning).

Necropsy:

No abnormal changes attributable to the test substance were noted in either sex. In one female of the low dose group (5000 ppm), a unilateral small eyeball was noted. This isolated finding was considered to be incidental, as there was no dose-response.

Organ weights:

At the dose level of 20000 ppm, statistically significant decreases in absolute organ weights were observed in both sexes for brain, thymus and spleen, and in females for the uterus. In addition, absolute spleen weights were also statistically significantly decreased in the mid dose male pups (10000 ppm). Furthermore, statistically significant decreases in relative weights of thymus and spleen were noted in both sexes at 20000 ppm.

Statistically significant increases of relative brain weights (in mid and high dose males as well as in high dose females) and of relative uterus weights (in high dose females) were considered to result from excessive lower body weight at necropsy.

The results of the range finding study for reproduction toxicity study are given in the following table.

Table 52: Results of the preliminary reproduction range finding study in rats

	Males				**Females**			
Diet concentration (ppm)	**0**	**5000**	**10000**	**20000**	**0**	**5000**	**10000**	**20000**
Parental animals (F0)	**Body weight (g)**							
Before mating (Day 0)	217.0	215.9	217.1	216.2	162.6	159.1	160.2	161.2
Before mating (Day 28)	324.4	328.9	320.0	**302.2***	211.8	205.2	**199.3****	**201.6***
Gestation Day 20	379.3	383.4	374.4	**346.8***	329.4	318.4	**312.5***	**284.3****
Lactation Day 21	(Day 62)	(Day 62)	(Day 62)	(Day 62)	267.5	277.4	267.8	**244.3****
	Food consumption (g/animal/day)							
Before mating (Day 7)	17.6	18.0	17.0	**13.1****	13.6	**11.7****	**12.0***	**10.1****
Before mating (Day 28)	18.4	18.7	18.4	18.1	13.9	13.7	**12.2****	13.0
Gestation Day 20	17.4	17.9	18.0	17.3	19.7	18.2	19.3	**16.7****
Lactation Day 21	(Day 62)	(Day 62)	(Day 62)	(Day 62)	61.8	57.7	**50.3***	**36.8****
	Necropsy findings							
Liver dark brownish change	0	0	0	10	0	0	1	8

Liver enlargement	0	0	5	10	0	0	1	7
Thyroid enlargement	0	1	1	4	0	0	1	1
	Organ weights							
Terminal body weight (g)	379	383	374	**347***	268	277	268	**244****
Liver (g)	10.8	**13.4****	**14.7****	**16.3****	12.0	14.1	**16.4****	**19.8****
Thyroid (mg)	22.4	**29.9***	**30.1***	**33.4****	18.2	18.3	20.8	19.6
Ovary (mg)					93.9	84.7	82.6	**64.8****
Uterus (g)					0.503	0.490	**0.301****	**0.229****
	Histopathology							
Number of animals examined	10	10	10	10	10	10	10	10
Liver: brown pigment in bile duct, focal (Grade 1-3)	0	0	1	6	0	0	5	9
Liver: brown pigment deposition in perilobular hepatocytes (Grade 1)	0	0	0	3	0	0	2	6
Liver: periductular inflammatory cell infiltration, focal (Grade 1)	0	0	1	4	0	0	3	8
Liver: eosinophilic foci of altered hepatocytes (Grade1)	0	0	0	1	0	0	0	1
Liver: centrilobular hypertrophy, hepatocyte (Grade1-3)	0	4 (Grade1)	10 (Gr. 1-3)	10 (Gr. 1-3)	0	6 (Gr. 1-2)	10 (Gr. 1-2)	10 (Gr. 2-3)
Liver: Bile duct proliferation (Grade 1-2)	0	0	0	1	0	0	3	5
Ovary: decreased vacuolation in interstitial gland (Grade1)	-	-	-	-	0	0	1	7
Uterus atrophy (Grade 1)	-	-	-	-	0	0	2	10
Thyroid: diffuse hypertrophy of follicular cells (Grade 1)	0	0	2	5	0	0	0	0
	Reproductive performance							
Copulation Index (%)	100	100	100	100	100	100	100	100
Fertility Index (%)	100	100	100	100	100	100	100	100
Litter examination	**Pup Viability**							
Implantation sites per dam					13.5	12.1	11.5	11.0
Offspring at birth per dam					13.0	10.4	10.0	10.5

Live offspring at birth per dam					13.0	10.4	10.0	**10.1***
Viability index on Day 0					100	100	100	**96.50***
Weaning index					100	100	100	95
Offspring (F1)	**Pup body weight (g)**							
Day 0	5.7	6.0	5.9	5.6	5.4	5.8	5.6	5.2
Day 21	52.5	49.4	**45.0***	**26.6****	50.5	47.7	**43.4***	**25.5****
	Pup organ weights on weaning							
Terminal body weight (g)	53.1	49.9	**44.9****	**26.3****	50.5	48.8	**44.3***	**25.8****
Brain (g)	1.459	1.437	1.438	**1.258****	1.424	1.417	1.390	**1.228****
Thymus (mg)	202.2	194.1	172.2	**75.6****	208.2	206.4	174.0	**80.1****
Spleen (g)	0.248	0.210	**0.194***	**0.091****	0.228	0.210	0.189	**0.088****
Uterus (mg)	-	-	-	-	36.91	37.98	31.38	**25.18****

Grade: 1 Minimal; 2 Mild; 3 Moderate
* $p < 0.05$ in comparison to controls, ** $p < 0.01$ in comparison to controls

CONCLUSION:

In a dose range finding reproduction study, groups of 10 male and 10 female Han Wistar rats (F0 generation) were exposed to mandestrobin (S-2200 TG) at dietary concentrations of 0 (control), 5000, 10000 or 20000 ppm for 4 weeks before mating, and throughout mating, gestation, and lactation periods.

Regarding the general toxicological effects on parental animals, suppression of body weight and body weight gain was noted in the males of the high dose group (20000 ppm). In females, body weight, body weight gain and food consumption were significantly decreased at 10000 ppm and above. Pathological examination revealed brown pigment in the bile duct and in perilobular hepatocytes, followed by inflammatory cell infiltration into the periductular region and proliferation of bile ducts in the 10000 ppm group and above. Dark brownish change and enlargement of the liver was noted at the necropsy in the 10000 ppm group and above. Significant liver weight increase was observed in males at ≥ 5000 ppm and in females at ≥ 10000 ppm. These liver changes were considered to be toxicologically relevant.

In females, decreased vacuolation in the interstitial gland in the ovary and atrophy of the uterus were noted in the 10000 ppm group and above. Ovary weights were decreased at 20000 ppm, and uterus weight decreased in the 10000 ppm group and above. The changes in the ovary and uterus were considered to be test substance-related changes; however, there were no treatment-related changes in reproductive function such as mating ability, fertility, pregnancy, parturition, or nursing behaviour in females.

Regarding the effects on offspring, the viability index on Day 0 decreased in the 20000 ppm group. Suppressed body weight and body weight gain was noted in both sexes in the 10000 ppm group at weaning and at 20000 ppm throughout the lactation period. Pathological examination

revealed reduced spleen weights at ≥ 10000 ppm and reduced organ weights of brain, thymus and uterus in the 20000 ppm group.

The no-observed-adverse-effect-level (NOAEL) for parental systemic toxicity is set at 5000 ppm for females (equivalent to 317 mg/kg bw/d; mean test substance intake during gestation) based on decreased body weight and food consumption and on liver effects at 10000 ppm. The dose level of 5000 ppm (244 mg/kg bw/day as the lowest substance intake after mating) is considered to be a lowest-observed-adverse-effect-level (LOAEL) for males, as liver weight increase greater than 20% was observed at this dose level.
A reproductive NOAEL of 20000 ppm (equivalent to 1229 mg/kg bw/d; mean test substance intake in females during gestation) was derived, due to the absence of effects on reproduction at the highest dose level tested.
The NOAEL for offspring systemic toxicity is proposed to be set at 5000 ppm (equivalent to 317 mg/kg bw/d; mean test substance intake during gestation) based on significantly lower body weight of pups at weaning and reduced spleen weights at 10000 ppm.

A dose of 10000 ppm was concluded to be appropriate as the high dose for the two-generation reproduction study of S-2200 TG in rats, at which some toxic signs should occur in at least F0 dams, but is also expected to ensure sufficient survival over two generations.

Reference:	Two-Generation Reproduction Toxicity Study of S-2200 TG in Rats
Author(s), year:	Matsuura, I.; 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd., Report No. ROT-0064
Guideline(s):	Japanese MAFF 12-Nousan-8147 (2000), US EPA OPPTS 870.3800 (1998), OECD TG 416 (2001)
GLP:	Yes (laboratory certified by National Authority)
Deviations:	Due to the Great East Japan Earthquake occurring at 14:46 on March 11, 2011, the utilities (electricity, steam, water) were shut down, which caused deviations from the protocol. However, there were no related abnormalities in any observation or investigation, including reproductive function and pathological findings, and all animals were judged to be healthy. Therefore, it was judged to have had no adverse effect on the reliability of the study. In addition, a number of further minor deviations from the protocol occurred, which were all considered as not relevant for the validity of the study.
Validity:	Yes

MATERIAL AND METHODS:

Test Material:	S-2200 Technical Grade (S-2200TG)
Lot/Batch:	ST-0811G
Purity:	93.4% (by Certificate of Analysis)
Test animals:	
Species:	Rat
Strain:	Wistar, BrlHan: WIST@Jcl (GALAS)
Age:	6 weeks at start of administration
Weight at start:	Males 174-199 g, females 122-147 g
Source:	CLEA Japan, Inc.
Diet:	CRF-1 (Oriental Yeast Co., Ltd.) sterilized by gamma rays

ad libitum

This study was conducted to assess the effects of mandestrobin (S-2200 TG) on the reproductive functions, such as oestrus cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring when administering S-2200 TG orally via diet over two generations in rats.

Animal assignment and treatment:

The dose levels were selected based on the results of a dose range-finding study (dietary dose levels: 0, 5000, 10000, 20000 ppm, study No. B091037). A dose of 10000 ppm was selected as the highest dose for the main study, at which some toxic signs occurred in parental animals. The intermediate and low doses were set at 3000 and 1000 ppm, respectively, with a common ratio of about 3.

Table 53: Study Design for the 2-generation reproduction study

Group	Diet concentration (ppm)	Number of F0 animals*[1]		Number of F1 animals*[2]	
		Male	Female	Male	Female
1	0	26	26	25	25
2	1000	26	26	26	26
3	3000	26	26	26	26
4	10000	26	26	24	24

*[1]: Number of treated animals
*[2]: For F1 animals, 1 male and 1 female were selected at weaning (postnatal Day 21) from each litter in numerical order of the animal number as animals for post-weaning examination (F1 parental animals) and subjected to administration.

Groups of 26 male and 26 female young adult rats were allocated to treatment groups (F0 generation). These rats were treated every day for 10 weeks before mating for both sexes, through the mating period until the day of necropsy in males, and through the mating, gestation, and lactation periods until the day of weaning of F1 offspring (Day 21 of lactation) in females (total administration period: 17 weeks for males and 16-18 weeks for females). F1 animals were treated every day from weaning (postnatal Day 21) for 10 to 12 weeks before mating for both sexes, and thereafter, in the same way as F0 animals (total administration period: 17-18 weeks for males and 16-18 weeks for females). Non-delivering females, however, were treated until 26 days after copulation (the day of necropsy).

Diet preparation and analysis:

Based on the results of analysis of storage stability of the test compound in the diet, the diet was prepared 9 times at a frequency of once within every 5 weeks (expiration period: 8 weeks). Slightly more than the required amount of the test compound was ground using a porcelain mortar and pestle. The test compound and an appropriate amount of the weighed basal diet were mixed and ground with a compact mill as the primary mixed diet. Then, the primary mixed diet was added with a portion of the weighed basal diet at a total amount of approximately 2 kg, and

mixed with a table top universal mixer to make a secondary mixed diet. Finally, the secondary mixed diet and the remaining weighed basal diet were mixed with a V or W type mixer, according to the amount of the preparation, for about 30 min. The prepared diet was subdivided into portions for about a week in plastic bags. For the control group, the basal diet was packed and sealed in a plastic bag by the supplier and was used without any preparation.

The mixed diet for the analysis of storage stability of the test compound in the diet was prepared as follows. A primary mixed diet prepared by the same method described above was added with a portion of the weighed basal diet, and it was mixed by shaking in a plastic bag for 1 min to make a secondary mixed diet. The secondary mixed diet and the remainder of the weighed basal diet were mixed and shaken in a plastic bag in the same manner. In order to confirm the stability of the test compound in the diet, the concentration of the test compound was analysed for the 1000 and 10000 ppm dietary formulations after storage for 2, 4, and 8 weeks under refrigeration (5.2 to 6.4°C) in a dark place, and for 2 weeks under the animal room conditions (21.9 to 22.9°C). Analytical samples (n=1) were collected from 3 points (top, middle, and bottom layers, one sample from each layer) of each of the dietary formulations, and analysed. As a result, the 1000 and 10000 ppm dietary formulations were confirmed to be stable under the tested conditions.

With respect to the homogeneity of the test substance in the mixed diet, samples from the top, middle, and bottom layers (n=2) were analysed for each dose level at the first preparation, and the coefficient of variation (C.V. value) of the mean concentration was confirmed to be ≤ 10%.

Observation and examination of parental animals (F0 and F1)

Clinical observations:

Cage-side observations were carried out once a day, and animals were palpated at least once a week (the day of body weight measurement).

Body weight:

Body weights were measured on the first day of dosing, and once a week thereafter until necropsy including the day of the necropsy for males, and until copulation for females. Copulated females were weighed on Days 0 (day of copulation), 7, 14, and 20 of gestation and on Days 0 (day of parturition), 4, 7, 14, and 21 of lactation (Day 21 = day of the necropsy). Body weight gain was calculated on the basis of the body weight on the first day of dosing during the pre-mating and mating periods, Day 0 of gestation during the gestation period, and Day 0 of lactation during the lactation period.

Food consumption

Food consumption was measured once a week by cage unit from the first day of dosing to the last week before the necropsy for males, except for the period of cohabitation with a female, and to 10 weeks after the start of dosing for females. For copulated females, food consumption was individually measured on Days 0, 7, 14, and 20 of gestation and days 0, 4, 7, 14, and 21 of lactation.

Chemical intake was calculated based on the concentrations of the test compound in diet (nominal concentration) and body weight and food consumption of each animal.

Oestrus cycle:

The oestrous cycle was examined by collecting vaginal smears every day in the morning for 3 weeks from 7 weeks after the start of dosing in the pre-mating period (F0 female: 13 weeks of age; F1 female: 10 weeks of age) and from the day after the start of mating, until copulation was confirmed in the mating period. From the results of the examination in the pre-mating period, the mean oestrous cycle length for each female and the incidence of females with irregular oestrous cycles were calculated. Females that had an oestrous cycle length other than 4 to 6 days were judged as having irregular oestrous cycles.

Reproductive function:

After the pre-mating administration period (F0 animals: 10 weeks, F1 animals: 10 to 12 weeks), males and females of the same group were cohabited day and night on one-to-one basis avoiding siblings in F1 animals for up to 14 days (first mating). From the day after the start of mating, vaginal smears were collected every day in the morning, while observing for the presence of vaginal plugs, and the smears were examined microscopically to determine oestrous cycle stage and the presence of sperm. Copulation was confirmed by the presence of a vaginal plug or sperm in the vaginal smears, and the day on which the evidence of copulation was found was designated as Day 0 of gestation.

The following indices were calculated:

- Days until copulation: Number of days from the start of mating to detection of copulation
- Number of oestrus stages without copulation
- Copulation index (%): (Number of animals with successful copulation/Number of animals paired) × 100
- Fertility index (%): (Number of pregnant animals/Number of animals with successful copulation) × 100

Sperm analysis:

At the necropsy of adult males, the right testis and cauda epididymis were removed, weighed, and examined for the following parameters:

Sperm motility

For all males of each group, a part of the cauda epididymis was incised to collect sperm. The sample was suspended in Medium 199 supplemented with 1% bovine serum albumin (BSA). The percentage of motile sperm was determined by analysing approximately 200 sperm cells per animal with an automatic sperm analyser.

Sperm morphology

Sperm smears stained with eosin Y were prepared from the sperm suspensions prepared for the sperm motility analysis. The incidence of abnormal sperm in the control and the high dose (10000 ppm) groups was determined by counting the morphologically abnormal sperm out of 200 complete sperm with head and a tail. The abnormal sperm was morphologically classified as sperm without hook, sperm with banana-like head, amorphous head, or folded midpiece, or others. The incidence of tailless sperm was determined by counting the sperm separated into the head and tail out of 100 sperm. The lower dose groups in both generations were not examined,

because no effects of the test compound were detected in F0 or F1 males in the high dose group (10000 ppm).

Spermatid count and sperm count

After the sperm motility analysis, the testis and cauda epididymis were stored frozen at -20°C or below (-27.9 to -21.6°C). Before the analysis, the organs of F0 and F1 males in the control and 10000 ppm groups were thawed at room temperature and homogenized in purified water to make stock solutions for the determination of spermatid and sperm counts. The sperm heads in the homogenates were counted with the automatic sperm analyser to determine the number of spermatids per gram of testis (homogenization-resistant spermatid) and the number of sperm per gram of cauda epididymis. The lower dose groups in both generations were not examined, because no effects of the test compound were detected in F0 or F1 males in the high dose group (10000 ppm).

Observation of parturition and nursing:

For parturition, the dams were observed twice a day from Day 21 to Day 25 of gestation. The females that delivered their litter completely by 16:00 were judged as dams giving birth on that day. The females that did not deliver by 25 days after copulation were judged as "non-delivery." For nursing, the dams were observed once a day for maternal behaviour, including lactation, nest building, and cannibalism, until Day 21 of lactation. At necropsy on Day 21 of lactation, the uterus was removed and examined for the number of implantations. The animals judged "non-delivery" were examined 26 days after copulation in the same way. The uterus without visible implantation sites was immersed in a 10% ammonium sulfide solution to detect the presence of implantation sites. The following indices were calculated based on these results:

- Gestation length: Number of days from Day 0 of gestation to the day of parturition
- Gestation index (%): (Number of females with live offspring/Number of pregnant females) × 100
- Birth index (%): (Number of offspring born alive/Number of implantations) × 100

Pathological examination:

Necropsy:

Upon completion, or near completion, of the scheduled necropsy of females on Day 21 of lactation, males were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the head, thoracic, and abdominal organs/tissues were examined macroscopically (F0 males: 5 weeks after the mating period, F1 males: 6 weeks after the mating period). Females were necropsied in the same manner as males at the following time: on Day 21 of lactation for dams delivered, 26 days after copulation for non-delivery females (2 F0 females and 3 F1 females), and 14 days after the mating period for the non-copulated female (1 F0 female only). For dams sacrificed on Day 21 of lactation, vaginal smears were collected in the morning on the day of the necropsy and examined for the oestrous cycle stage. The age at necropsy was 23 to 24 weeks for F0 males, 20 to 24 weeks for F0 females, 20 to 21 weeks for F1 males, and 18 to 21 weeks for F1 females.

Organ weight:

At the scheduled necropsy of males and dams after weaning, the following organs were weighed: Brain, pituitary, thyroid, liver, kidney, adrenal, spleen, testis, epididymis (whole and cauda),

prostate (ventral lobe), seminal vesicle (including coagulating gland), ovary, uterus (including cervical region).

Relative organ weights (body weight ratio) were calculated based on each body weight at necropsy. The pituitary and thyroid were weighed after fixation in 10 vol% phosphate-buffered formalin. The bilateral organs were weighed individually and calculated as a sum.

Histopathological examination:

The following organs/tissues of all animals were fixed and preserved in 10 vol% phosphate-buffered formalin (apart from the testes which were pre-fixed in Bouin's fluid for more than 2 hours and then transferred to 10 vol% phosphate-buffered formalin): Brain, pituitary, thyroid, thymus, liver, kidney, adrenal, spleen, testis, epididymis, seminal vesicle, coagulating gland, prostate (ventral lobe), ovary, uterine tube, uterus (including cervical region), vagina, mammary gland (female), gross lesions.

For the histopathological examination, the following organs/tissues of all males and females in the control and high dose (10000 ppm) groups were stained with haematoxylin and eosin (H.E.) by standard methods, and were examined microscopically: Pituitary, adrenal (bilateral), testis (unilateral), epididymis (unilateral), seminal vesicle (bilateral), coagulating gland (bilateral), prostate (ventral lobe), ovary (bilateral), uterine tube (bilateral), uterus (including cervical region), and vagina.

In addition, the liver of males and females and the thyroid of males in the F0 and F1 generations, and the kidney of F0 females were examined in the same way, because effects of the test substance were suspected from the results of organ weight measurement.

For the epididymis, longitudinal sections of the unilateral head, body, and caudal portion were prepared and examined. The ovary of F1 females was cut at the maximum diameter, embedded in paraffin (left and right ovaries separately), and sectioned. The paraffin-embedded sections were subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA), and the number of small follicles, medium-sized follicles, and large follicles was counted (according to the classification by Pedersen *et al*.).

Since treatment-related changes were seen in liver of top-dose F0 and F1 males and females, in thyroid of top dose F0 and F1 males, and in adrenals of top dose F1 females, these organs of F0 and F1 animals in the 1000 and 3000 ppm groups were also examined in the same manner. In addition, liver sections from 2 males and 2 females each in the control and 10000 ppm groups in the F0 and F1 generations were stained with Schmorl, and Hall and Berlin blue for the differentiation of the brown pigment observed in the liver of the animals in the 10000 ppm group. Liver sections stained with H.E. were also examined using a polarizing lens and a fluorescence microscope to verify whether the brown pigments had birefringent and red autofluorescence or not. For the special stainings of the liver, animals that had severe changes in the 10000 ppm group were selected.

Observation and examination of offspring (F1 and F2)

Litter examinations:

The day of birth was designated as postnatal Day 0.

On postnatal Day 0, the newborns were examined for the number of offspring (live or stillborn), sex, and presence of external anomalies. After that, the pups were observed daily for clinical signs and mortality until postnatal Day 21. On postnatal Day 4, litter size was randomly adjusted

to 8 pups (equal sex ratio, in principle). Litters with less than 8 pups were maintained as they were. The pups culled at the litter size adjustment were subjected to necropsy. From the numbers of live offspring on postnatal Days 0, 4, 7, 14, and 21 (weaning day), the following indices were calculated:

- Live birth index (%): (Number of offspring born alive/Number of offspring born) × 100
- Viability index on Day 4 (%): (Number of offspring alive on Day 4/Number of offspring born alive) × 100
- Viability index on Day 7 (%): (Number of offspring alive on Day 7/Number of live offspring after culling) × 100
- Viability index on Day 14 (%): (Number of offspring alive on Day 14/Number of live offspring after culling) × 100
- Weaning index [viability index on Day 21] (%): (Number of live weanlings/Number of live offspring after culling) × 100

For F1 animals, 1 male and 1 female were randomly selected from each litter (the first offspring number of male and female pups selected randomly at the litter size adjustment) on postnatal Day 21 and became the parental animals for the second generation (F1 parental animals). Other F1 pups and all F2 pups were necropsied on postnatal Day 21.

Body weight:

The offspring were weighed individually on postnatal Days 0, 4, 7, 14, and 21. The body weight gain was calculated by litter unit on the basis of the body weight at birth before the litter size adjustment, and by each offspring on the basis of the body weight on postnatal Day 4 after the litter size adjustment.

Sexual maturation:

The F1 offspring were examined for the day of vaginal opening from postnatal Day 27 for females and for the day of preputial separation (cleavage of the balanopreputial gland) from postnatal Day 35 for males. Body weights were measured on the corresponding days.

Pathological examination:

Necropsy:

Pups culled at the litter size adjustment on postnatal Day 4 were euthanized by exsanguination from the abdominal aorta under anaesthesia, and the head, thoracic, and abdominal organs/tissues were examined macroscopically. All of the other pups, except for F1 animals selected as parents for the next generation, were necropsied in the same manner on postnatal Day 21. The following organs of pups subjected to the organ weight measurement (one pup/sex/litter) and organs/tissues showing gross abnormality were fixed in 10 vol% phosphate-buffered formalin: Brain, thymus, spleen, testis, epididymis, seminal vesicle, prostate, ovary, uterus, and vagina.

The pups that died before the litter size adjustment, except for cannibalized ones, were examined for the presence of external anomalies and the whole bodies were preserved in 10 vol% phosphate-buffered formalin. The pups that died after the litter size adjustment could not be necropsied, because all were cannibalized by their dams.

Organ weight:

At necropsy of F1 pups on postnatal Day 21, 1 male and 1 female were selected from each litter (in numerical order of the offspring number in each litter) for organ weight measurement (limited to: brain, thymus, spleen, uterus). Relative organ weights (body weight ratio) were calculated based on individual body weight at necropsy.

Statistics:

Offspring data obtained before weaning were analysed on the basis of litter mean values, except for the sex ratio. The body weights and food consumption data from non-pregnant females after copulation were excluded from the analysis.

The metric data were analysed by multiple comparison tests for statistical significance. The homogeneity of variance was tested first by Bartlett's test. When the variance was homogeneous, the one-way analysis of variance was performed; when heterogeneous, the Kruskal-Wallis test was used. When a significant inter-group difference was found, Dunnett's or the Dunnett-type multiple comparison test was used. For some of the data, the Kruskal-Wallis test was applied first, and when a significant inter-group difference was found, the Dunnett-type multiple comparison test was used. However, for the comparison of spermatid count, sperm count, and ovarian follicle count between the control and 10000 ppm groups, the homogeneity of variance was tested by the F test, and when the variance was homogeneous, Student’s t test was performed for the statistical comparison; when heterogeneous, the Aspin-Welch t test was used. In addition, Wilcoxon’s rank sum test was used for the comparison of the incidence of abnormal sperm (morphologically abnormal sperm and tailless sperm) between the control and 10000 ppm groups. The count data were analysed by Wilcoxon’s rank sum test for histopathological findings and by Fisher’s exact probability test for the others. The significance level was set at 5% for all statistical analyses. All statistical analyses were performed with the Toxicological Data Processing System (MiTOX, Mitsui Zosen Systems Research Inc.).

Statistical analyses were performed on the items listed below. The analyses were not conducted on the clinical observations.

- *Multiple comparison test:* Body weight, body weight gain, food consumption, organ weight, number of implantations, number of offspring, number of live offspring, oestrous cycle length
- *Kruskal-Wallis test and Dunnett-type multiple comparison test:* Days until copulation, number of oestrus stages without copulation, gestation length, birth index, live birth index, viability index on Days 4, 7, and 14, weaning index, incidence of offspring with external anomalies, sexual maturation (vaginal opening, preputial separation), sperm motility
- *F test and t test:* Spermatid count, sperm count, ovarian follicle count
- *Wilcoxon’s rank sum test:* Incidence of abnormal sperm, histopathological findings, percentage of ovarian follicles
- *Fisher’s exact probability test:* Copulation index, fertility index, gestation index, sex ratio (male/female), incidence of females with irregular oestrous cycles, incidence of dams with externally abnormal offspring, necropsy findings

FINDINGS:

Chemical intake:

The mean test substance intakes in the treated groups are shown in the following table (Table 54). The mean intakes during the pre-mating period in F1 animals were approximately 1.5-fold higher for both sexes compared with those in F0 animals, reflecting the higher intake in the growth period after weaning. The mean intake of males after mating and that of females during both the gestation and lactation periods were approximately the same between F0 and F1 animals for each treated group. The mean intake during the gestation period was similar to that in the pre-mating period, whereas during lactation it was more than 2-fold higher than during gestation in both F0 and F1 females.

Table 54: Mean test substance intakes (mg/kg bw/d)

Sex	**Doses** **Period of treatment**	**1000 ppm (Group 2)**	**3000 ppm (Group 3)**	**10000 ppm (Group 4)**
F0 generation				
Males	Pre-mating period	56.15	166.3	559.1
	After mating period	43.27	132.0	452.1
Females	Pre-mating period	62.48	195.3	628.5
	Gestation period	60.19	186.2	602.8
	Lactation period	162.8	511.0	1634.6
F1 generation				
Males	Pre-mating period	84.73	254.5	881.2
	After mating period	47.77	145.7	511.7
Females	Pre-mating period	90.11	274.9	929.3
	Gestation period	65.68	200.3	672.0
	Lactation period	163.4	504.6	1687.5

Clinical observations:

There was no mortality among parental animals, or among F1 animals selected for rearing.

F0 animals

No abnormal clinical signs were observed in males or females of any group throughout the observation period, including the gestation and lactation periods of pregnant females.

F1 animals

No treatment-related abnormal clinical signs were observed in males or females in any treated group throughout the observation period.

Before weaning, death was observed sporadically in a few pups in all groups, including the control group, mainly up to postnatal Day 4. There was no death after the litter size adjustment on postnatal Day 4, except for 1 female (#60425-6) that died on postnatal Day 13 in the 10000 ppm group. Most of the pups that died were cannibalized by their dams. Trauma or internal haemorrhage was observed in one pup of the 1000 ppm group and in one pup of the 3000 ppm group during the period before postnatal Day 4. Moreover, loss of suckling was seen on postnatal Days 2 and 3 in pups of one dam (#50221) that showed loss of retrieving on Day 3 of lactation in the 1000 ppm group, resulting in body weight loss or remarkably reduced body weight gain in these pups. However, these findings were judged to be not treatment-related because there was no dose-dependency in their incidences or because of their low occurrence.

F2 animals

No treatment-related abnormal clinical signs were observed in males or females in any treated group throughout the observation period.

Death was observed sporadically in a few pups in all dose groups, including the control group, mainly up to postnatal Day 4. There was no death after the litter size adjustment on postnatal Day 4, except for 1 male and 1 female (#20411-43, #70404-58) that died on postnatal Day 5 in the 10000 ppm group. Most of the pups that died were cannibalized by their dams.

Body weight:

F0 animals

In the 10000 ppm group, body weight gain was suppressed in males, with significant differences in body weights and body weight gain throughout the observation period. In females, although there were no significant differences in body weights or body weight gain during the pre-mating period, the body weights were suppressed in the gestation and lactation periods, and significant differences were noted in body weight gain on Day 14 of gestation and in body weights on Days 7 and 14 of lactation. However, the body weight gain during the lactation period was almost comparable to that in the control group up to Day 14 of lactation, and a significantly higher value was observed on Day 21 of lactation[1].

In the 1000 and 3000 ppm groups, body weights were comparable to those in the control group in males and females throughout the observation period, including the gestation and lactation periods of pregnant females, with no significant differences in body weights or body weight gain.

F1 animals

In the 10000 ppm group, although birth weights were almost the same as those in the control group, postnatal body weight gain was suppressed in males and females before weaning, and statistically significant differences were noted in both sexes in body weight and body weight gain from postnatal Days 7 to 21. After weaning, suppressed body weight gain continued in males until the terminal necropsy, with significant differences in body weight and body weight gain at almost all measurement points. In females, significantly lower body weights also continued after weaning. However, a significant difference in body weight gain was observed only on postnatal Day 28, and the difference in the body weights from the control group diminished with time, with no significant differences observable after postnatal day 63 in the pre-mating period. In the gestation and lactation periods, the body weights were again suppressed, and significant differences were noted in body weights on Day 7 of gestation and on Days 4 to 14 of lactation. However, the body weight gain during the gestation and lactation periods was almost comparable to that in the control group up to Day 14 of lactation, and a significantly higher value was observed on Day 21 of lactation.

[1] Regarding the increased body weight gain of F0 and F1 dams on lactation Day 21 at 10000 ppm compared to control: It is well known that the body weights of lactating dams increase due to the development and engorgement of the mammary gland in the early stage of lactation and decrease in the later period, because the pups consume the diet during the last week of lactation which triggers involution of the mammary gland, with precipitous decline after weaning of their pups. In the 10000 ppm group, the body weight gain of the pups was remarkably suppressed, suggesting prolongation of lactation in these pups and delay in the physiological involution of the mammary gland in their dams, which resulted in suppression of the physiological decrease of maternal body weights in the later stage of the lactation period. Therefore, the increased maternal body weight gain on lactation Day 21 at 10000 ppm was considered to be due to growth retardation in their pups, and was judged to be not toxicologically relevant.

In the 1000 and 3000 ppm groups, birth weights, postnatal body weights, and body weight gain in males and females were comparable to those in the control group throughout the observation period, including the gestation and lactation periods. Significantly higher values in body weight gain were observed on postnatal Days 28 and 35 in the 3000 ppm group and in body weights on Day 21 of lactation in the 1000 and 3000 ppm groups; however, they were judged to be incidental because of a transient change with no dose-dependency.

F2 animals

Although birth weights were almost the same as those in the control group, postnatal body weight gain was suppressed in males and females at 10000 ppm, and significant differences were noted in both sexes in body weights and/or body weight gain from postnatal Days 7 to 21.

In the 1000 and 3000 ppm groups, birth weights, postnatal body weights, and body weight gain were comparable to those in the control group in males and females throughout the observation period, with no significant differences in body weight or body weight gain.

Food consumption

F0 animals

In the 10000 ppm group, food consumption decreased significantly in males from Days 28 to 63 of dosing. In females, although there were no significant differences during the pre-mating or gestation period, a significantly lower value was noted on Day 7 of lactation.

In the 1000 and 3000 ppm groups, food consumption was almost comparable to that in the control group in males and females throughout the observation period, including the gestation and lactation periods of pregnant females. Significantly higher food consumption was observed on Day 7 of dosing in the 3000 ppm group; however, it was judged to be incidental because of a transient change with the lack of dose-dependency.

F1 animals

In the 10000 ppm group, significantly lower food consumption was noted in males on postnatal Days 28 and 35 of dosing; however, there were no significant differences thereafter. In females, significantly lower food consumption was also observed sporadically, with statistical significance on postnatal Days 28, 70, and 91 during the pre-mating period as well as on Day 7 of gestation and on Days 4 to 14 of lactation. In the 1000 and 3000 ppm groups, food consumption was comparable to that in the control group in males and females throughout the observation period, including gestation and lactation periods, with no significant differences.

Oestrus cycle:

In the F0 generation as well as in the F1 generation, no treatment-related changes were observed in the oestrous cycles in females of any treated group. Almost all females in each group had an oestrous cycle of 4 to 5 days, and no significant differences were found in the mean oestrous cycle length. There were no females with irregular oestrous cycles in any group, including the control group.

Reproductive performance:

As a result of the first mating, all mating pairs in each group in both generations showed evidence of copulation by 14 days after the start of mating, except for 1 pair in the 10000 ppm group in the F0 generation. Therefore, the test compound was judged to have no effect on the mating ability, and the mating was terminated without the second mating in both generations.

F0 animals

No treatment-related changes were observed in the mating ability or fertility in males or females of any treated group. All the mating pairs of each group, except for one pair in the 10000 ppm, copulated during the first or second oestrus stage of females within 13 days after the start of mating. There were no significant differences in the copulation index, in the number of oestrus stages without copulation or in the days until copulation between the control and treated groups. There was only 1 non-pregnant female in the control group and in the 10000 ppm group, respectively, and no significant difference was found in the fertility index between the control and treated groups.

The uncopulated pair in the 10000 ppm group did not copulate during 3 oestrus cycles in the mating period. In addition, 1 female each in the 1000 and 10000 ppm groups showed continuous dioestrus after the start of mating; however, both females returned to oestrus by 12 or 13 days after the start of mating, and copulated and conceived. They were judged to be incidental because only one case was observed in each group with no dose-dependency.

F1 animals

No treatment-related changes were observed in the mating ability or fertility in males or females of any treated group.

All the mating pairs of each group copulated during the first or second oestrus stage of females within 5 days after the start of mating, and there were no significant differences in the copulation index, in the number of oestrus stages without copulation or in the days until copulation between the control and treated groups. There were only 1 and 2 non-pregnant females in the 1000 and 3000 ppm groups, respectively, and no significant difference was found in the fertility index between the control and treated groups.

Sperm analysis:

In both the F0 and the F1 generation, no treatment-related changes suggesting effects on spermatogenesis were detected in any treated group. All parameters, including the sperm motility, spermatid counts, sperm counts, and the incidences of abnormal sperm and tailless sperm were comparable between the control and treated groups, with no significant differences. Furthermore, no abnormalities were observed in males that had no copulation or did not impregnate their mating partners, except for 1 male in the control group of the F0 generation, which showed low sperm motility of 40%.

Observation of parturition and nursing:

F0 animals

No treatment-related changes were detected in parturition or nursing in any treated group. All dams in each group delivered normally between Days 21 and 23 of gestation and no significant differences were found in the gestation length, number of implantations or offspring born alive, birth index, or gestation index between the control and treated groups.

In the observation of nursing, loss of retrieving was observed in 1 dam in the 1000 ppm group on Day 3 of lactation; however, it was judged not to be treatment-related because only one case was observed with the lack of dose-dependency in the incidence. There were no abnormalities in nursing behaviour in the other dams of any group.

F1 animals

No treatment-related changes were detected in parturition or nursing in any treated group. All dams in each group delivered between Days 21 and 23 of gestation, except for 1 dam that delivered on Day 24 of gestation in the control group. No abnormalities were observed in parturition in any group, including the dam that delivered on Day 24 of gestation in the control group.

There were no significant differences in the number of implantations or offspring born alive, birth index, or gestation index between the control and treated groups, although the gestation length in the 10000 ppm group (21.8 days) was significantly shorter than in the control group (22.2 days). However, there was no treatment-related change in gestation length of F0 dams. In F1 dams, the difference from the control group in gestation length was extremely small and the gestation length of all dams in the 10000 ppm group was 21 or 22 days, which is within the normal range (21 to 23 days) of this strain. Moreover, the mean gestation length in the 10000 ppm group was almost equal to the lower limit of the historical control range of the test facility (mean length: 21.9-22.2 days, range of individual length: 21-23 days, 2002-2009), while that in the control group was equal to the upper limit of the historical control range. Individually, 5 out of 24 dams had a gestation length of 21 days in the 10000 ppm group, whereas in the control group none of the dams delivered before Day 22 of gestation and 1 dam had a gestation length of 24 days, which has not been observed in the historical control data of the test facility. In F0 dams of the control group, 2 out of 25 dams had a gestation length of 21 days, while further 2 of them had already started parturition at 16:00 on Day 21 of gestation. However, since parturition was not completed at 16:00, they were judged as dams giving birth on Day 22 of gestation (data not shown), indicating that the occurrence of parturition on Day 21 of gestation in F1 dams of the 10000 ppm group was almost the same as that in F0 dams in the control group. Therefore, the significant difference observed in the gestation length in the 10000 ppm group was considered to be a variation within the normal range, and was judged to be of no toxicological significance.

In the observation of nursing, there were no abnormalities in nursing behaviour in any dam of any group.

Pup development:

Viability:

In F1 and F2 pups, no treatment-related changes were detected in viability in any treated group. All parameters in each treated group, including the number of offspring born and born alive, sex ratio, live birth index, viability index on Days 4, 7, and 14, and weaning index, were comparable to those in the control group, with no significant differences.

Moreover, there was no death after weaning in any F1 group, including the control group

External examination:

There were no external anomalies in F1 or F2 pups of any group, including the pups that died.

Sexual maturation (F1 animals):

At 10000 ppm, preputial separation (cleavage of balanopreputial gland) in males and vaginal opening in females showed a significant delay of 1.6 and 1.5 days on average (males: Day 42.7, females: Day 32.5) compared to the control group (males: Day 41.1, females: Day 31.0), respectively. The body weights in the 10000 ppm group on the corresponding days were lower than those in the control group in both sexes (% of the control value: males, 96.1%, females, 94.1%), and a significant difference was observed in females.

In the 1000 and 3000 ppm groups, both, the days of preputial separation in males and vaginal opening in females, were comparable to those in the control group, with no significant differences. Moreover, no significant differences were found in body weights in males or females on the corresponding days.

Pathological examination:

Necropsy:

F0 animals

Dark brownish change of the liver was noted in 24 females in the 10000 ppm group. Enlargement of the liver was noted in 20 females in the 10000 ppm group. In the non-copulated and non-pregnant females, there were no treatment-related changes.

Greenish change in the cortex of the kidney was noted in 5 females in the 10000 ppm group. However, there were no histopathological changes attributed to the necropsy finding.

Other changes were considered to be incidental, because of the lack of dose-response and/or their pathological nature.

F1 animals

F1 pups culled on postnatal Day 4:

There were no abnormal findings in offspring of any group, including the control group.

F1 pups necropsied at weaning:

No treatment-related abnormal findings were detected in males or females in any treated group. The single pup that died after the litter size adjustment (one female in the 10000 ppm group) could not be examined due to cannibalization by the dam.

F1 parental animals:

Liver: Dark brownish change of the liver was noted in 20 females in the 10000 ppm group. Enlargement of the liver was noted in 6 females in the 10000 ppm group. In the non-pregnant females, there were no treatment-related changes.

The other changes were considered to be incidental due to the lack of dose-response and/or their pathological nature. Furthermore, enlargement of the thyroid observed in 1 and 2 males at 3000 and 10000 ppm, respectively, was judged to be incidental, because vacuolation of the follicular cells was confirmed histopathologically in all of the males in the 10000 ppm group, which is known to occur spontaneously in this strain of rats.

F2 pups

F2 pups culled on postnatal Day 4:

There were no abnormal findings in offspring of any group, including the control group.

F2 pups necropsied at weaning:

No treatment-related abnormal findings were found in males or females in any treated group.

The pups that died after the litter size adjustment (one male and one female in the 10000 ppm group) could not be examined due to cannibalization by their dams.

Organ weight:

F0 animals

In males, significant increases in the absolute and relative weights of the thyroid and liver were noted in the 3000 ppm group and above. Significantly higher relative weights of the brain and seminal vesicle were observed in the 10000 ppm group. However, the absolute weights of both organs were comparable to those in the control group, with no significant differences; therefore, these changes were considered to be due to the lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

In females, significant increases in the absolute and relative weights of the liver were noted in the 1000 ppm group and above, as were increases in the absolute and relative weights of the kidney and decreases in the absolute and relative weights of the uterus in the 10000 ppm group. Moreover, the ovary weights in the 10000 ppm group tended to be lower than those in the control group and a significant difference was observed in the relative weights. Regarding the oestrous stage at the necropsy in females, the distribution of each stage was similar among all groups. Most of the females in each group showed dioestrus and the others exhibited proestrus or oestrus, with no constant trend among the groups.

F1 animals

F1 pups necropsied at weaning:

Significant decreases in the absolute and relative weights of the spleen were noted in males in the 3000 and 10000 ppm groups. In addition, at 10000 ppm, significantly lower absolute weights were observed in the thymus in both sexes and the spleen and uterus in females, as were significantly higher relative weights of the brain in both sexes in the 10000 ppm group. However, the changes observed only in either absolute or relative weights and the corresponding absolute or relative weights of these organs were almost the same as those in the control group; therefore, they were considered to be due to the lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

F1 Parental animals:

In males, significant increases in the absolute and relative weights of the thyroid and liver were noted in the 10000 ppm group. Absolute weights of the liver in the 3000 ppm group also tended to be higher than those in the control group and a significant difference was observed in the relative weights. In addition, significantly higher relative weights of several other organs (brain, spleen, kidney, adrenal, testis, seminal vesicle, and epididymis) were observed in the 10000 ppm group. However, the absolute weights of these organs were comparable to those in the control group, with no significant differences; therefore, these changes were considered to be due to the

lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

In females, significant increases in the absolute and relative weights of the liver were noted at 3000 and 10000 ppm. The absolute and relative weights of the liver in the 1000 ppm group were also higher than those in the control group, with a significant difference in the absolute weights. In addition, absolute and relative ovary weights were significantly decreased in the 10000 ppm group. Furthermore, significantly lower values were observed in the absolute weights of the brain in the 10000 ppm group and relative brain weights in the 1000 and 3000 ppm groups, in the relative weights of the pituitary in the 3000 and 10000 ppm groups, and in the relative weights of the left adrenal in the 3000 ppm group. However, they were changes observed only in either absolute or relative weights, with the lack of clear dose-dependency; therefore, they were judged to be not treatment-related or of no toxicological significance.

Regarding the oestrous stage at the necropsy in females, the distribution of each stage was similar among all groups. Most of the females in each group showed metoestrus or dioestrus and the others exhibited proestrus or oestrus, with no constant trend among the groups.

F2 pups

Significant decreases in the absolute and relative weights of the spleen were noted in males and females in the 10000 ppm group. Moreover, the spleen weights of females in the 3000 ppm group also tended to be lower than those in the control group, and a significant difference was noted in the relative weights. In addition, significantly lower absolute weights were observed in the thymus in males and females, as well as significantly higher relative weights of the brain in both sexes in the 10000 ppm group. However, these changes were only observed only in either the absolute or relative weight and the corresponding relative or absolute weights of these organs were similar to the control group; therefore, they were considered to be due to the lower body weights at the necropsy in this group, and were judged to be of no toxicological significance.

Histopathological examination:

F0 animals

Liver: Minimal to moderate brown pigment in the bile duct/periportal area was observed in 9 males and 20 females in the 10000 ppm group. The brown pigments were considered to be porphyrin pigments, because the pigment stained dark blue with Schmorl and negative for Hall and Berlin blue, and had birefringent according to an examination using a polarizing lens. It had no clear red autofluorescence by a fluorescence microscope, tentatively attributed to the microscope conditions not being optimised. At 10000 ppm, minimal brown pigment deposition in the perilobular hepatocyte was observed in 4 females, minimal focal periductular inflammatory cell infiltration was observed in 4 males and 10 females, and minimal to moderate proliferation of the bile duct was observed in 12 females. Minimal to moderate diffuse hypertrophy of hepatocytes was observed in 6 females at 1000 ppm, in 15 males and 17 females at 3000 ppm, and in 24 males and all females at 10000 ppm.

Thyroid: Minimal diffuse hypertrophy of the follicular cells in the thyroid was observed in 4 males in the 10000 ppm group.

In the non-copulated and non-pregnant females, minimal brown pigment in the bile duct/periportal area and minimal diffuse hypertrophy of hepatocytes were observed in 1 and 2 females in the 10000 ppm group, respectively. The other changes were considered to be

incidental, because of the lack of dose-response and/or their pathological nature.

F1 animals

Liver: Minimal to moderate brown pigment in the bile duct/periportal area was observed in 10 males and 11 females in the 3000 ppm group, and in all animals in the 10000 ppm group. The brown pigment stained dark blue with Schmorl and negative for Hall and Berlin blue, and had birefringence according to an examination using a polarizing lens. It had no clear red autofluorescence by a fluorescence microscope, tentatively attributed to the microscope conditions not being optimised. Minimal brown pigment deposition in the perilobular hepatocytes was observed in 5 females in the 10000 ppm group. Minimal focal periductular inflammatory cell infiltration was observed in 3 females in the 3000 ppm group, and in all animals in the 10000 ppm group. Minimal to moderate proliferation of the bile duct was observed in 16 females in the 10000 ppm group. Minimal to mild diffuse hypertrophy of hepatocytes was observed in 4 females at 1000 ppm, in 6 males and 18 females at 3000 ppm, and in 23 males and all females at 10000 ppm.

Adrenals: Minimal hypertrophy of the cortical cells in the fascicular zone was observed in 5 females in the 10000 ppm group.

Thyroid: Although histopathological examination of thyroid was extended to all groups in view of the suspicion that treatment-related changes could occur as seen among F0 males, there were no treatment-related changes in the thyroid of F1 males. In one male in the 3000 ppm group, there were no histopathological changes attributed to the enlargement of the thyroid detected at necropsy.

In the non-pregnant females, there were no treatment-related changes.

The other changes were considered to be incidental, because of the lack of dose-response and/or their pathological nature.

F1 Ovarian follicle count

A significant increase in the number of medium-sized follicles was noted in the 10000 ppm group. In addition, although there was no significant difference, the number of large follicles also tended to increase in the 10000 ppm group. However, the number of small follicles, which include primordial follicles, and the total number of ovarian follicles in the 10000 ppm group were comparable to those in the control group, with no significant differences. Moreover, the percentage of ovarian follicles at each developmental stage in the 10000 ppm group was almost equal to that in the control group, indicating no effects on oogenesis.

In addition, there were no treatment-related changes suggesting impairment of the ovarian function in any reproductive observations, including oestrus cycles and the number of implantations, and no histopathological changes were detected in the ovary in this group. Consequently, it was judged to be incidental and of no toxicological significance.

Table55: Summary of key findings in the F0 generation in the two-generation reproduction toxicity study

	Males				**Females**			
Diet concentration (ppm)	**0**	**1000**	**3000**	**10000**	**0**	**1000**	**3000**	**10000**
Body weight (g)								

Before mating (Day 0)	185.8	185.7	185.9	185.8	134.6	135.8	135.9	135.8
Before mating (Day 70)	393.8	391.2	386.2	**369.9****	221.1	225.4	225.1	220.4
Gestation Day 20	438.7 (Day 119)	437.7 (Day 119)	436.1 (Day 119)	**415.0**** (Day 119)	326.1	327.0	329.2	324.3
Lactation Day 21					268.1	272.7	273.8	273.9
Body weight gain (g)								
Before mating (Day 0-70)	208.0	205.5	200.3	**184.1****	86.5	89.7	89.2	84.6
Gestation (Day 0 – 7)	252.9 (Day 0-119)	252.0 (Day 0-119)	250.2 (Day 0-119)	**229.2**** (Day 0-119)	24.2	21.0	21.8	20.8
Gestation (Day 0 – 14)					48.2	44.0	43.9	**42.7****
Gestation (Day 0 – 20)					103.3	101.0	103.3	102.0
Food consumption (g/animal/day)								
Before mating (Week 10)	17.8	17.5	17.3	17.0	12.9	12.3	12.4	12.5
Before mating (mean over Weeks 4-9)	18.0	17.8	17.4	**16.6****	12.7	12.4	12.9	12.4
Lactation (Day 7)					41.8	39.1	41.5	**36.0****
Lactation (Day 14)					47.6	45.2	48.3	44.3
Lactation (Day 21)					60.4	58.2	60.0	58.8
Reproductive Performance								
Number of pairs					26	26	26	26
Mating index (%)					100	100	100	96.2 (25/26)
Mean precoital time (days)					2.9	3.2	2.5	3.6
Fertility index (%)					96.2 (25/26)	100	100	96.0 (24/25)
Gestation index (%)					100	100	100	100
Gestation length (days)					22.1	22.0	22.0	22.0
Implantations (mean)					13.0	12.2	13.2	13.0
Offspring born alive					12.2	11.7	12.2	12.3
Birth index (%)					93.78	95.86	92.64	95.40
Necropsy gross findings								
Number of animals examined	26	26	26	26	25	26	26	24
Liver: Dark brownish change	0	0	0	0	0	0	0	**24****
Liver: Enlargement	0	0	0	0	0	0	0	**20****

Kidney: Greenish change, cortex	0	0	0	0	0	0	0	**5***
Organ weights								
Liver (g)	12.65	13.04	**14.12****	**15.53****	10.42	**11.45***	**12.24****	**17.24****
Relative liver weight (% bw)	2.87	2.97	**3.23****	**3.73****	3.89	**4.21***	**4.47****	**6.30****
Kidney (g)	2.536	2.545	2.555	2.499	1.987	1.992	1.961	**2.095****
Relative kidney wt. (% bw)	0.577	0.580	0.585	0.599	0.742	0.731	0.717	**0.766****
Thyroid (mg)	23.75	24.04	**25.29****	**28.97****	20.98	21.90	21.67	21.55
Relative thyroid wt. ($x10^{-3}$%)	5.45	5.48	**5.78****	**7.02****	7.85	8.00	7.93	7.88
Ovary (mg)					91.34	90.35	97.48	82.33
Relative ovary wt. ($x10^{-3}$%)					34.14	33.21	35.66	**30.06***
Uterus (g)					0.45	0.54	0.51	**0.38***
Relative uterus wt. (% bw)					0.167	0.198	0.187	**0.137****
Histopathology								
Liver (no. examined)	26	26	26	26	25	26	26	24
Brown pigment, bile duct/periportal	0	0	0	**9**** (grade 1)	0	0	0	**20**** (grade 1-3)
Brown pigment deposition in perilobular hepatocytes	0	0	0	0	0	0	0	**4*** (grade 1)
Focal periductular inflammatory cell infiltration	0	0	0	**4*** (grade 1)	0	0	0	**10**** (grade 1)
Proliferation of bile ducts	0	0	0	0	0	0	0	**12**** (grade 1-3)
Diffuse hypertrophy of hepatocytes	0	0	**15**** (grade 1)	**24**** (grade 1-2)	0	**6*** (grade 1)	**17**** (grade 1)	**24**** (grade 1-3)
Thyroid (no. examined)	26	26	26	26	0	0	0	0
Diffuse hypertrophy of follicular cells	0	0	0	**4*** (grade 1)				

Grade: 1 Minimal; 2 Mild; 3 Moderate
* $p < 0.05$ in comparison to controls, ** $p<0.01$ in comparison to controls

Table 56: Summary of key findings in the F1 and F2 generations in the two-generation reproduction toxicity study

	Males				**Females**			
Diet concentration (ppm)	**0**	**1000**	**3000**	**10000**	**0**	**1000**	**3000**	**10000**
F1 animals								
Body weight (g)								
Postnatal Day 0	5.8	5.8	5.8	5.8	5.5	5.5	5.4	5.5
Postnatal Day 7	16.2	15.9	16.3	**14.7***	15.7	15.5	15.7	**14.2***
Postnatal Day 14	32.5	32.0	32.3	**28.0****	31.6	31.2	31.4	**27.1****
Postnatal Day 21	52.0	51.1	50.3	**43.8****	50.3	49.5	48.5	**41.9****
Before mating (Day 91)	390.7	391.6	381.7	**353.0****	224.0	229.5	225.7	211.8
Gestation Day 20	465.5	464.1	452.5	**428.0****	340.8	349.5	346.3	328.9
Lactation Day 21	(Day 147)	(Day 147)	(Day 147)	(Day 147)	277.4	**289.9***	**290.1***	279.0
F1 pup development								
Day of cleavage of balanopreputial gland	41.1	41.8	42.0	**42.7****				
Day of vaginal opening					31.0	30.9	31.0	**32.5***
Bodyweight (g) at cleavage of balanopreputial gland / vaginal opening	173.96	181.38	179.62	167.17	94.96	96.46	95.50	**89.33***
Reproductive Performance								
Number of pairs					25	26	26	24
Copulation index (%)					100	100	100	100
Day of conceiving					2.6	2.6	2.3	2.0
Fertility index (%)					100	96.2	92.3	100
Gestation index (%)					100	100	100	100
Gestation length (days)					22.2	22.0	22.0	**21.8****
Implantations (mean)					13.8	13.5	13.1	13.2
Offspring born alive					13.0	12.9	12.5	12.3
Birth index (%)					93.96	95.29	95.44	93.04
Necropsy gross findings – parental animals								
No. of animals examined	25	26	26	24	25	25	24	24
Liver: Dark brownish change	0	0	0	0	0	0	0	**20****
Liver: Enlargement	0	0	0	0	0	0	0	**6****

Organ weights – at weaning								
Body weight (g)	52.0	51.4	50.5	**44.0****	50.6	49.8	48.8	**41.6****
Spleen (g)	0.266	0.248	**0.232***	**0.199****	0.252	0.250	0.232	**0.200****
Thymus (mg)	204.1	199.0	203.4	**167.8****	208.8	206.2	208.5	**172.1****
Uterus (mg)	-	-	-	-	40.38	40.83	42.70	**34.60***
Organ weights – parental animals								
Body weight (g) at necropsy	466.6	466.0	455.0	**427.3****	277.4	**289.9***	**290.1***	279.0
Liver weight (g)	13.86	14.00	14.62	**15.97****	11.14	**12.30****	**13.33****	**16.96****
Relative liver weight (% bw)	2.97	3.00	**3.22****	**3.74****	4.01	4.25	**4.59****	**6.10****
Thyroid weight (mg)	25.32	25.12	26.02	**32.41***	23.61	22.06	20.62	21.03
Relative thyroid wt. ($x10^{-3}$%)	5.45	5.39	5.75	**7.72****	8.49	7.65	7.12	7.55
Ovary weight (mg)	-	-	-	-	97.10	95.83	98.25	**84.40****
Relative ovary wt. ($x10^{-3}$%)	-	-	-	-	35.09	33.03	33.84	**30.28****
Brain (g)	2.093	2.092	2.050	2.072	1.890	1.890	1.862	**1.843***
Histopathology								
Liver (no. examined)	25	26	26	24	25	25	24	24
Brown pigment, bile duct/periportal	0	0	**10**** (grade 1)	**24**** (grade 1-2)	0	0	**11**** (grade 1)	**24**** (grade 1-3)
Brown pigment deposition in perilobular hepatocytes	0	0	0	0	0	0	0	**5*** (grade 1)
Focal periductular inflammatory cell infiltration	0	0	0	**24**** (grade 1)	0	0	3	**24**** (grade 1)
Proliferation of bile ducts	0	0	0	0	0	0	0	**16**** (grade 1-3)
Diffuse hypertrophy of hepatocytes	0	0	**6*** (grade 1)	**23**** (grade 1-2)	0	**4*** (grade 1)	**18**** (grade 1)	**24**** (grade 1-2)
Adrenal (no. examined)	25	0	0	24	25	25	24	24
Hypertrophy of cortical cell in the fascicular zone	0			0	0	0	0	**5*** (grade 1)
F2 animals								
Body weight (g)								
Postnatal Day 0	5.8	5.7	5.9	5.8	5.5	5.5	5.6	5.4

Postnatal Day 7	16.0	15.8	15.9	15.0	15.5	15.3	15.5	**14.3***
Postnatal Day 14	32.9	31.7	32.4	**29.6****	32.1	31.0	31.8	**28.5****
Postnatal Day 21	53.5	51.6	52.2	**46.3****	51.4	50.3	50.4	**44.5****
Organ weights								
Thymus weight (mg)	212.0	204.9	201.5	**182.5****	210.7	209.0	205.3	**179.0****
Spleen weight (g)	0.273	0.253	0.245	**0.209****	0.274	0.262	0.234	**0.203****

Grade: 1 Minimal; 2 Mild; 3 Moderate
* $p < 0.05$ in comparison to controls, ** $p < 0.01$ in comparison to controls

CONCLUSION:

Mandestrobin (S-2200 TG) was administered orally to male and female rats via diet at doses of 1000, 3000, and 10000 ppm over two generations, and the effects on the reproductive function of parental animals and development of the next generations were assessed.

Regarding the general toxicological effects on parental animals, suppressed body weight gain and reduced food consumption were noted in males and in females in the 10000 ppm group in both F0 and F1 generations.

Pathological examination revealed treatment-related changes in the liver of both sexes. At necropsy, dark brownish change and enlargement of the liver were noted in F0 and F1 females in the 10000 ppm group. Liver weights increased in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the histopathological examination the following findings were observed: Brown pigment in the bile duct/periportal area (in F0 animals at 10000 ppm and in F1 animals at ≥ 3000 ppm), focal periductular inflammatory cell infiltration (in F0 males and females and F1 males at 10000 ppm and in F1 females at ≥ 3000 ppm), and brown pigment deposition in the perilobular hepatocyte and proliferation of the bile duct (in F0 and F1 females in the 10000 ppm group). Brown pigment in the bile duct, the periportal area, and the perilobular hepatocyte were considered to be primary changes by the test substance. Periductular inflammatory cell infiltration and proliferation of the bile ducts were considered to be secondary changes occurred by the pigmentation. These primary and secondary changes in F1 animals showed an increase in incidence compared to F0 animals.

Diffuse hypertrophy of the hepatocyte was also observed in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. It is known that hepatocellular hypertrophy accompanied by increased liver weights is an adaptive change associated with induction of the hepatic microsomal drug metabolizing enzymes, and it is considered not to be adverse in the absence of histopathological damage indicative of hepatotoxicity and relevant clinical chemistry changes. In the 1000 ppm group, only hepatocellular hypertrophy and increased liver weights were observed in F0 and F1 females, without any other change. Therefore, the changes in the liver observed in females in the 1000 ppm group were considered to be an adaptive change, and of no toxicological significance.

Furthermore, increases in the thyroid weights were observed in F0 males at ≥ 3000 ppm and in F1 males at 10000 ppm, and hypertrophy of the follicular cell of the thyroid was observed in some F0 males in the 10000 ppm group. The increase in hepatic drug metabolizing enzyme activity is known to cause an increase of the clearance of thyroid hormones in hepatocyte, resulting in hypertrophy in the thyroid follicular cells by negative feedback. As the thyroid effects were

observed only at dose levels higher than that resulting in hepatocellular hypertrophy, the hypertrophy of thyroid follicular cells in F0 males in the 10000 ppm group is considered to be secondary to the increased in hormonal turnover and the changes in the liver.

Treatment-related hypertrophy of cortical cells in the fascicular zone was observed in the adrenals in some F1 females in the 10000 ppm group. The effects of the test substance in F1 females were only slightly more severe than in F0 females and are possibly an adaptive change in response to stress.

In addition, decreases were observed in ovary weights in F0 and F1 females and uterus weights in F0 females in the 10000 ppm group, however, in absence of any histopathological changes.

Furthermore, greenish change in the cortex of the kidney was observed in some F0 females at necropsy in the 10000 ppm group, with increased organ weights. However, there were no histopathological changes related to the necropsy finding and increased organ weights in F0 females, nor were there any changes in the kidney of F1 females or F0 or F1 males. In the 13-week repeated oral dose toxicity study in rats, no abnormal changes in the kidney of females were detected in necropsy or histopathological findings or blood parameters of the renal function. Therefore, the changes in the kidney observed in F0 females were considered to be of no toxicological significance.

Based on liver effects (weight increase, diffuse hypertrophy of hepatocytes) observed in female parental animals of the F0 and F1 generation, the lowest dose of 1000 ppm was considered a LOAEL by the experts (corresponding to 60.19 mg/kg bw day mean substance intake of female F0 animals during gestation).

No parental NOAEL could be derived from this study.

Regarding the effects on the reproductive function, there were no treatment-related changes in mating ability, fertility, pregnancy, gestation length, parturition, or nursing behaviour, nor were there any changes in the oestrous cycle or sperm parameters (sperm motility, spermatid counts, sperm counts, and incidences of morphological abnormal sperm and tailless sperm) in either generation. Therefore, the no-observed-adverse-effect level (NOAEL) for the reproductive effects is set at the highest dose level tested in this study of 10000 ppm (559 mg/kg bw/day; mean substance intake of F0 males during pre-mating period).

Regarding the effects on offspring, postnatal body weight gain was suppressed in both sexes of F1 and F2 offspring in the 10000 ppm group. Since both F0 and F1 dams in the 10000 ppm group had reduced food consumption during the gestation and/or lactation period, with lower maternal body weights, the postnatal growth retardation observed in this group may be due to undernourishment of their dams.

In addition, lower spleen weights at weaning were noted in F1 males in the 3000 ppm group, in which no change was found in postnatal body weight gain, and in all F1 and F2 animals at 10000 ppm. The lower spleen weights of F1 animals at weaning completely recovered to the control level at adult in both sexes even in the 10000 ppm group, suggesting a transient retardation in growth. Moreover, both the absolute and relative spleen weights of F1 and F2 animals were within the historical control range in the test facility, indicating slight changes.

A slight delay in sexual maturation was found in both sexes (mean difference from the control group: 1.5 days for vaginal opening in F1 females and 1.6 days for preputial separation in F1 males) in the 10000 ppm group. However, there were no changes suggesting effects on the reproductive function in any observations or examinations, including oestrous cycle, sperm parameters, and histopathological findings. Therefore, the slight delay in sexual maturation in both sexes was considered to be related to the growth retardation.

Under the conditions of this study, the NOAEL for effects on offspring is considered to be 1000 ppm (56 mg/kg bw/d; mean substance intake of F0 males during pre-mating period).

4.11.1.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.11.2 Developmental toxicity

4.11.2.1 Non-human information

Rats:

Reference:	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rat
Author(s), year:	Rhodes, J.; 2009a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0009
Guideline(s):	This study was not conducted to any specific regulatory guideline, but was conducted to support OECD TG 414, EPA OPPTS 870.3700 and Japanese MAFF 12-Nousan-8147 Teratogenicity studies (2-1-18).
GLP:	No (non-regulatory study, for which a claim of GLP compliance has not been made; however, conducted in accordance with current GLP requirements)
Deviations:	No
Validity:	Yes (supplementary study)

MATERIAL AND METHODS:

Test Material: S-2200 Technical Grade (S-2200 TG)
Lot/Batch: ST-0811G
Purity: 93.4%

Test animals:
Species: Rat
Strain: Wistar, Crl:WI(Han)
Age at mating: 9 weeks
Weight at mating: 186.7 – 224.0 g
Source: Charles River (UK) Ltd, Margate

Diet: SQC Rat and Mouse Breeder Diet No 3 *ad libitum*

The objective of the study was to assess the effects of mandestrobin (S-2200 TG) on the pregnancy of rats when administered orally by gavage, in order to select dose levels for a subsequent study of prenatal development.

Investigations were limited to maternal growth and food consumption, and pregnancy parameters to ensure that dose selection in future study would result in sufficient pups for a full evaluation of developmental toxicity.

Animal assignment and treatment:

Female rats were cohabited with male rats on a one to one basis. Once evidence of mating was seen (presence of vaginal plug *in situ*, or other evidence of mating if necessary), 7 presumed-pregnant females were allocated to each dosage group (0, 250, 500 and 1000 mg/kg bw/d).

The day on which evidence of mating was seen, was designated Day 0 of gestation.

Dose preparation, analysis and administration:

The test article was formulated as a suspension in aqueous 0.5% (w/v) methylcellulose and was stirred continuously before and throughout dosing. Solutions of the test article in the vehicle were prepared for each concentration daily.

Test and control articles were administered orally to presumed-pregnant female rats by gavage once daily from Days 6 to 19 of gestation. The females were sacrificed on Day 20 of gestation for macroscopic examination and examination of litters. Individual dose volumes were adjusted according to the most recent bodyweight, determined daily. Control animals received 0.5% w/v methylcellulose solution.

Clinical observations:

All animals were examined twice daily to detect dead or moribund animals. All animals were examined at least once daily for signs of ill health or overt toxicity. Any abnormalities of appearance or behaviour or other signs of reaction to treatment were recorded and a detailed individual record was maintained of the clinical condition of each animal on the days of body weight recording. Additionally, animals were observed immediately after dosing and at 0.5, 1, 2 and 4 hours post dose for signs of reaction to treatment.

Body weight and food consumption:

The body weight of each female was recorded on Days 4, 6, 7, 8, 9, 12, 15, 17, 19 and 20 of gestation.

Food consumption was recorded for Days 4 to 5, 6, 7, 8, 9 to 11, 12 to 14, 15 to 16, 17 to 18 and 19 of gestation.

Sacrifice and pathology:

At the scheduled necropsy on Day 20 of gestation, females were sacrificed by cervical dislocation following isoflurane anaesthesia and examined macroscopically. All tissues were discarded. Gross lesions were retained in 10% neutral buffered formalin.

Uterine/implantation data:

The ovaries and uteri were removed and examined and the following data recorded: pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations (subdivided into live foetuses, early intrauterine deaths, late intrauterine deaths and dead foetuses).

The uterus of any apparently non-pregnant female was immersed in a 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live foetuses were killed by a subcutaneous injection of sodium pentobarbitone.

Individual foetal and placental weights were recorded and foetuses were examined externally and sexed.

The foetuses were stored in 10% neutral-buffered formalin. On completion of data recording, all foetuses were discarded as there was no effect of treatment.

Statistics:

All variables were analysed with a two-sided risk except where stated below.

Body weight gains, Day 20 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test was used to assess the equality of variances among the groups. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Foetal weights, placental weights, the percentage of male foetuses and the numbers of corpora lutea, implantations and foetuses per female were analysed using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the corrected body weight on day 20 as covariate. This analysis depends on the assumption that the relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed and this showed no evidence of heterogeneity ($p \geq 0.01$).

The proportions of females affected by pre- and post-implantation loss, by early and late intrauterine deaths and the number of litters affected by variations and malformations were analysed using the Cochran-Armitage test for dose-response and Fisher’s exact test for pairwise

comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Some variables were not analysed due to all animals having the same value or there being too few distinct values for meaningful analysis.

FINDINGS:

Mortality and clinical signs:

There were no unscheduled deaths during the study, and no treatment-related significant adverse clinical signs were observed. Piloerection was seen in all treated animals from Day 6 of gestation until the next day. On Day 7 of gestation piloerection was seen in all treated animals at half an hour post-dosing and in 2, 3 and 5 animals at doses of 250, 500 and 1000 mg/kg bw/day, respectively, for up to 1 hour post-dosing only. This typical finding in rodents was considered likely to be a reaction to the dosing procedure itself rather than evidence of toxicity.

Body weight, body weight gain and food consumption:

Mean body weight and mean body weight gain throughout the study were unaffected by treatment. There were no treatment-related effects on mean food consumption throughout the study.

Necropsy findings:

There were no significant adverse findings at necropsy.

Uterine/implantation data:

There were no adverse effects observed at examination of the uterus. Mean gravid uterus weight showed no effect of treatment. The number of pregnancies was 6, 7, 6 and 7 in the control, low dose, intermediate dose and high dose groups, respectively. The mean numbers of corpora lutea, implantations and the mean incidence of pre- and post-implantation loss showed no adverse effects of treatment.

The mean litter size was unaffected by treatment.

Foetal data:

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean foetal weight; differences between the groups did not show a dose-related trend and were therefore not considered to be adverse.

In the dose group of 250 mg/kg bw/day, there were two malformed foetuses in two litters; one foetus had a severely reduced orbit of the eye, the other foetus had no patent anal opening. There was one malformed foetus in the dose group of 500 mg/kg bw/day; a severely displaced umbilical opening associated with absent muscle tissue. In animals dosed at 1000 mg/kg bw/day, the

incidence of the variation haematoma of the lower jaw was higher than in the control group, but this was an isolated finding and was not considered likely to be an adverse effect of treatment.

Overall, there was no effect of treatment on the mean incidence of external foetal variations and malformations.

Table 57: Key findings for dose range finding teratogenicity study in rats

Dose group (mg/kg bw/day)	**0**	**250**	**500**	**1000**
Number of females mated	7	7	7	7
Non-pregnant	1	0	1	0
Mortality	0	0	0	0
Number of females with live foetuses on Day 20	6	7	6	7
Maternal body weight on Day 20 (g)	306.9	317.1	312.3	310.3
Maternal weight gain from Days 6 to 20 (%)	37.7	43.5	42.9	41.4
Mean number of corpora lutea per female	13.2	12.7	12.8	14.4
Mean number of implantations per female	11.7	11.9	11.8	10.4
Mean number of foetuses	11.0	11.6	11.3	10.4
Mean placental weight (g)	0.44	**0.50***	0.45	**0.49***
Mean foetal weight (g)	3.64	3.73	3.84	3.67
Total number of foetuses showing malformations (% of foetuses examined)	0 (0)	2 (2.5)	1 (1.5)	0 (0)
Number of litters with external malformations	0/6	2/7	1/6	0/7

* $p < 0.05$ in comparison to controls

CONCLUSION:

In a preliminary study designed to investigate doses for further developmental toxicity testing, groups of 7 pregnant rats were administered daily doses of 0 (control), 250, 500 or 1000 mg/kg bw/day S-2200 TG from Days 6 to 19 of gestation. Administration of S-2200 TG by oral gavage to pregnant rats at dose levels up to 1000 mg/kg bw/day elicited no significant adverse effects of maternal or embryo-foetal toxicity. Under the conditions of this preliminary study, the highest dose level tested of 1000 mg/kg bw/day can be considered to be the maternal and developmental no-observed-adverse-effects-level (NOAEL).

Based on the results of this study, a top dose of 1000 mg/kg bw/day was recommended for a subsequent study of prenatal development.

Reference:	S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rat
Author(s), year:	Rhodes, J.; 2012a
Report/Doc.	Sumitomo Chemical Co., Ltd. Report No. ROT-0051

number:	
Guideline(s):	Japanese MAFF 12 Nousan 8147 Teratogenicity studies (2-1-18), EPA OPPTS 870.3700, OECD TG 414 (2001), EC 2004/73 B.31
GLP:	Yes (laboratory certified by National Authority)
Deviations:	No
Validity:	Yes

MATERIAL AND METHODS:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%

Test animals:

Species:	Rat
Strain:	Wistar, Crl:WI (Han)
Age at mating:	9-10 weeks
Weight at mating:	175.5 - 224.0 g
Source:	Charles River (UK) Ltd, Margate, UK
Diet:	SQC Rat and Mouse Breeder Diet No 3, Expanded, *ad libitum*

The objective of the study was to assess the effects of mandestrobin (S-2200 TG) on the pregnant rat and on the embryonic and foetal development when administered orally, by gavage.

Animal assignment and treatment:

On Day 3 of gestation the animals were assigned to four groups (24 animals/group) using a randomisation procedure based on body weight. Each group received dose preparations containing the control article (vehicle) or 100, 300 or 1000 mg/kg bw/day of test article. Treatment group positions in the room were assigned using a set of random number permutations. The test and control articles were administered orally, by gavage, to mated female rats daily from Days 6 to 19 of gestation, inclusive at approximately the same time each day. The females were maintained to Day 20 of gestation when they were killed and examined macroscopically. The foetuses were removed, killed and examined.

Dose preparation, analysis and administration:

Formulations of the test article in the vehicle were prepared weekly and dispensed as daily aliquots. The test article was formulated as a suspension in aqueous 0.5% w/v methylcellulose. The formulations were stored at room temperature in a sealed container. They were stirred on arrival at the animal room and were stirred continuously before and throughout dosing.

Stability was analysed in Covance Study Number 8202038 where concentrations of 2 and 250 mg/mL were found to be stable for up to 17 days when stored at room temperature. Homogeneity of samples from formulations prepared for the first and last day of dosing was analysed at Covance. The mean of the homogeneity results was used as the achieved concentration result.

The test and control articles were administered orally, by gavage at a dose volume of 5 mL/kg, to mated female rats daily from Days 6 to 19 of gestation, inclusive, at approximately the same time each day.

Clinical observations:

All animals were examined twice daily to detect dead or moribund animals. All animals were examined at least once daily for signs of ill health or overt toxicity. Any abnormalities of appearance or behaviour or other signs of reaction to treatment were recorded and a detailed individual record was maintained of the clinical condition of each animal on the days of body weight recording. Additionally, animals were observed immediately after dosing and at 0.5 and 1 hours post dose for signs of reaction to treatment.

Body weight and food consumption:

The body weight of each female was recorded on Days 3, 6, 7, 8, 9, 12, 15, 17, 19 and 20 of gestation.

Food consumption was recorded for Days 3-5, 6, 7, 8, 9-11, 12-14, 15-16, 17-18 and 19 of gestation.

Sacrifice and pathology:

At the scheduled necropsy on Day 20 of gestation, females were sacrificed by cervical dislocation following isoflurane anaesthesia. Post mortem examination included gross macroscopic examination of all internal organs, with gross lesions retained in relevant fixative.

Uterine/implantation data:

The ovaries and uteri were removed and examined, and the following data were recorded: pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations. Implantations were subdivided into: live foetuses, early intrauterine deaths, late intrauterine deaths and dead foetuses. Early intrauterine deaths were classified as those which showed decidual or placental tissue only. Late intrauterine deaths showed embryonic or foetal tissue in addition to placental tissue. Dead foetuses were classified as those which appeared to have died shortly before necropsy.

The uterus of any apparently non-pregnant female was immersed in a 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live foetuses were killed by a subcutaneous injection of sodium pentobarbitone and subsequently immersed in fixative.

Individual foetal and placental weights were recorded and foetuses were examined externally and sexed. Approximately one half of the foetuses in each litter, selected by systematic sampling, were dissected and the viscera were examined. They were then eviscerated and the carcasses were processed to stain the ossified skeleton by the Alizarin technique and cartilage processed to stain

using Alcian Blue. The skeletons were examined, preserved and stored in glycerol/propylene glycol.

The remaining foetuses were placed in Bouin's solution for at least two weeks to allow fixation and partial decalcification. At examination, the head was removed by a cut through the mouth, pharynx and the back of the head. The coronal sections of the head were examined. The remaining portion of the foetus was examined by dissection and was preserved, with the head sections, in 10% neutral buffered formalin and stored in plastic vials.

The dissection of the foetuses and the examination of the stained skeletons were performed using low power binocular magnification.

Foetal abnormalities were classified as malformations (rare and/or potentially lethal defects) and variations (commonly occurring non-lethal abnormalities).

Statistics:

The control group was taken as the baseline group with which the treated groups were compared. All variables were analysed with a two-sided risk except where stated below.

Body weight gains, Day 20 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test was used to assess the equality of variances among the groups. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Foetal weights, placental weights, the percentage of male foetuses and the numbers of corpora lutea, implantations and foetuses per female were analysed using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the corrected body weight on Day 20 as covariate. This analysis depends on the assumption that the relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed and this showed no evidence of heterogeneity ($p \geq 0.01$).

The proportions of females affected by pre- and post-implantation loss, by early and late intrauterine deaths and the number of litters affected by variations and malformations were analysed using the Cochran-Armitage test for dose-response and Fisher’s exact test for pairwise comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Some variables were not analysed due to all animals having the same value or there being too few distinct values for meaningful analysis.

The percentage of foetuses in each litter affected by variations and malformations were also analysed using the non-parametric methods, as previously described.

FINDINGS:

Mortality and clinical signs:

There were no unscheduled deaths during the study.

Clinical observations were unremarkable and showed no dose-related trend. The postdose observation mouth rubbing (and very occasionally salivation) was seen at 300 and 1000 mg/kg bw/day only immediately after dosing, but not at 0.5 and 1 hour post-dose. This postdose observation was seen with increasing frequency at 300 mg/kg bw/day from Day 9 of gestation onwards and at 1000 mg/kg/day from Day 7 of gestation onwards. This typical finding in rodents commonly observed in oral (gavage) prenatal developmental toxicity studies was considered likely to be due to taste aversion rather than evidence of toxicity. Two animals receiving 1000 mg/kg bw/day were thin on Day 20 of gestation and this finding was confirmed at necropsy.

Body weight, body weight gain and food consumption:

Group mean body weight gain and mean gravid uterus weight adjusted for body weight were both unaffected by treatment.

Over the first 3 days of dosing only mean food consumption at 1000 mg/kg/day was very slightly lower than control and this was statistically significant ($p < 0.05$, $p < 0.01$, $p < 0.05$ on Days 6, 7 and 8 of gestation, respectively). Over the entire dose period, mean food consumption was similar in all groups.

Necropsy findings:

Two animals receiving 1000 mg/kg bw/day were thin on Day 20 of gestation and this finding was confirmed at necropsy. Other findings at necropsy were typical for this strain of rat at these laboratories and showed no dose relationship and therefore are viewed as non-adverse.

Uterine/implantation data:

The number of pregnancies was 24, 23, 24 and 24 in the 0, 100, 300 and 1000 mg/kg bw/day dose groups respectively. The mean numbers of corpora lutea, implantations and the mean incidence of pre- and post-implantation loss showed no adverse effect of treatment. The number of dams with early intrauterine deaths or post-implantation loss at 1000 mg/kg bw/day showed an apparent statistical significance, however both mean number of early intrauterine deaths and mean percentage of post-implantation loss showed no dose relationship or statistical significance. Mean litter size was comparable in all dose groups. Therefore, this was not considered to be biologically significant. Historical control data for % intrauterine death (mean 0.4; range 0.2-0.6) and % post implantation loss (mean 3.4; range 1.8-5.0) were exceeded by the highest and lowest dose group in this study.

Foetal data:

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean foetal weight.

Malformations were noted in one foetus in the group receiving 300 mg/kg bw/day and in five foetuses from five litters in the group receiving 1000 mg/kg bw/day (detailed in table 58). The incidence and intergroup distribution of these foetal malformations do not indicate an adverse effect of treatment because there was no statistical significance or dose-related increase for the individual specific lesions. It is not considered appropriate to combine these diverse types of malformation for analysis. Overall, there was no adverse effect of treatment on the incidence of foetal variations and malformations, which were all within, or close to, expected ranges for this strain of rat.

Table 58: Key findings of the teratogenicity study in rats

Dose (mg/kg bw/day)	**0**	**100**	**300**	**1000**
Maternal bodyweight (g) on Day 20	303.2	300.2	309.9	300.4
Maternal weight gain, Days 6-20 (%)	41.4	39.2	43.3	38.4
Food consumption, Day 6 (g/rat/day)	19.1	17.8	18.6	**16.7***
Food consumption, Day 7 (g/rat/day)	20.5	19.2	19.4	**17.7****
Food consumption, Day 8 (g/rat/day)	20.2	19.7	20.3	**17.7***
Food consumption, Days 6-18 (g/rat/day)	21.4	20.8	21.0	20.5
Number of females mated	24	24	24	24
Non-pregnant	0	1	0	0
Pregnant (%)	24 (100)	23 (95.8)	24 (100)	24 (100)
Number of females with live foetuses	24	23	24	24
Mean number of early intrauterine deaths (number of dams affected)	0.3 (7)	0.8 (10)	0.4 (8)	0.7 (**14***)
% Post-implantation loss (number of dams affected)	2.8 (7)	8.6 (10)	4.4 (10)	6.9 (**14***)
Mean litter size	10.5	10.0	11.2	10.0
Mean foetal weight (g)	3.75	3.77	3.77	3.83
Mean placenta weight (g)	0.48	0.5	0.48	0.5
Sex ratio (% of male)	50.0	47.3	53.8	55.6
External or visceral malformations: number of foetuses affected (% of foetuses) / litter incidence	0	0	0	2 (0.7)/2
• Kidney, severely increased pelvic cavitation	0	0	0	2
External or visceral variations: number of foetuses affected (% of foetuses) / litter incidence	77 (30.9)/24	78 (35.7)/23	103 (37.4)/24	101 (42.4)/24

Skeletal malformations: number of foetuses affected (% of foetuses) / litter incidence	0	0	1 (0.8)/1	3 (2.9)/3
• Rib cartilage shortened	0	0	0	1
• Sternebrae, cleft xiphoid cartilage	0	0	0	1
• Vertebral cervical arch and centrum, additional ossification site fused	0	0	1	0
• Vertebral cervical arch, additional cartilaginous ventral plate fused	0	0	0	1
Skeletal variations: number of foetuses affected (% of foetuses) / litter incidence	122 (94.8)/24	108 (93.7)/23	128 (96.4)/24	120 (99.2)/24
Total number of foetuses with malformations, n (%)	0	0	1 (0.4)	5 (2.1)
Litter incidence	0	0	1	**5***

* p<0.05, ** p<0.01, *** p<0.001

Table 59: Historical control data for Han Wistar rats at Covance

Study number	**1[§]**	**2[§]**	**3[§]**	**4[$]**	**5[$]**	**6[$]**	**Mean**	**Range**
Early intrauterine deaths (mean number)	1.1	1.1	0.6	0.6	0.4	0.2	0.6	0.2-1.1
Post implantation loss (%)	10.3	14.1	4.8	5.0	3.4	1.8	6.5	1.8-14.1
External (visceral) malformations (%)	2 (1.3)	0 (0.0)	3 (1.5)	1 (0.5)	0 (0.0)	0 (0.0)	1	0-3
External (visceral) variations (%)	90 (52.2)	70 (40.8)	76 (32.0)	76 (30.3)	66 (25.5)	71 (31.0)	74.8	66-90
Skeletal malformations (%)	0 (0.0)	6 (6.7)	4 (8.3)	2 (1.8)	0 (0.0)	1 (0.8)	2.16	0-6
Skeletal variations (%)	76 (90.4)	88 (99.2)	99 (91.0)	97 (82.1)	113 (85.1)	88 (81.3)	93.5	76-113
Total number of malformations	2	6	7	3	0	1	3.1	0-7
Total number of variations	166	158	175	173	179	159	168.3	159-179

§ Animals supplied by Harlan $ Animals supplied by Charles River

Table 60: Cumulative foetal defect data for Han Wistar rats supplied by Harlan at Covance

Parameter	**Control group**
Number of foetuses examined for external/ visceral defects	571
Number of foetuses examined for skeletal defects	283
External (visceral) malformations (%)	5 (0.9)
External (visceral) variations (%)	236 (41.3)
Skeletal malformations (%)	10 (3.5)
Skeletal variations (%)	263 (92.9)

CONCLUSION:

Groups of 24 presumed-pregnant female rats were administered S-2200 TG at dose levels of 0, 100, 300, and 1000 mg/kg bw/day from Days 6 to 19 of gestation. All animals survived to the scheduled sacrifice on Day 20 and there were no treatment-related clinical signs or necropsy findings. Mean food consumption at 1000 mg/kg bw/day was slightly reduced over the first 3 days of dosing only. Mean body weight gain, and mean gravid uterus weight adjusted for body weight, were unaffected by treatment. There was no adverse effect of treatment on mean uterine/implantation data. Sex ratio, mean litter weight, mean placental weight and mean foetal weight were all unaffected by treatment and there was no adverse effect of treatment on the incidences of foetal variations or malformations at any dose level tested.

Based on these results, both the maternal no-observed-adverse-effect-level (NOAEL) and the embryo-foetal NOAEL were set at 1000 mg/kg bw/day.

Rabbits:

Reference:	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rabbits
Author(s), year:	Rhodes J, 2009b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0008
Guideline(s):	None stated (range finding study)
GLP:	No
Deviations:	Supplementary study
Validity:	Yes

MATERIAL AND METHODS:

Test Material:	S-2200 TG
Lot/Batch:	ST-0811G (Batch Specification 01)
Purity:	93.4%

Test animals:

Species:	Rabbit
Strain:	New Zealand White (Hsd:IfNZW)
Age at receipt:	18-22 weeks at mating
Weight:	3.10 - 3.90 kg at mating
Source:	Harlan (UK) Ltd, Loughborough
Diet:	Pelleted Harlan Teklad 2930C Rabbit Diet (Harlan Teklad, Madison, USA); available *ad libitum.*
Acclimation:	Time-mated by the supplier. Day of mating designated day 0 of gestation. Animals were delivered to the test laboratory on day 3 of gestation. On arrival the animals were examined and found to be in good health. An inspection by a veterinary surgeon before the start of treatment confirmed their suitability for experimental use.

The purpose of the study was to select proper dose levels of mandestrobin (S-2200 TG) for the main developmental study in rabbits.

Animal assignment and treatment:

On the day of arrival the time-mated and presumed pregnant animals were assigned to treatment groups each of 7 animals using a randomisation procedure based on body weight. The day of insemination was designated day 0 of gestation. Rabbits were dosed once each day during days 7 to 28 of gestation. The dose volume was 5 mL/kg.

Diet preparation, analysis and administration:

S-2200 TG was administered as a suspension in a 0.5% w/v aqueous methylcellulose solution, at dose levels of 0, 250, 500, and 1000 mg/kg bw/day. The test article was formulated as suspension in aqueous 0.5% (w/v) methylcellulose. Solutions of the test article in the vehicle were prepared daily.

Presumed-pregnant female rabbits were administered S-2200 TG by gavage at a dose volume of 5 mL/kg once daily during days 7 to 28 of gestation, and sacrificed on day 29 of pregnancy for macroscopic examination and examination of litters. Dose volumes were determined on the basis of the most recent bodyweight, determined daily. Control animals received 0.5% w/v methylcellulose solution.

Statistics:

Body weight gains, day 29 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test was performed to assess the equality of variances among the groups. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant. Foetal weights, placental weights, the percentage of male foetuses and the numbers of corpora lutea, implantations and foetuses per female were analysed using nonparametric methods.

The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error. Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the corrected body weight on day 29 as covariate. This analysis depends on the assumption that the relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed and this showed no evidence of heterogeneity ($p \geq 0.01$). The proportions of females affected by pre- and post-implantation loss and by early and late intrauterine deaths and the number of litters affected by variations and malformations were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Clinical observations:

All animals were checked twice daily for any mortalities. All animals were observed at least once daily for signs of reaction to treatment and/or symptoms of ill health. Additionally, animals were observed immediately after dosing and at 0.5, 1, 2 and 4 hours post dose.

Food consumption and body weight:

Individual food consumption was recorded daily from day 3 to 29 of gestation. Body weights were measured on days 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29 of gestation.

Sacrifice and pathology:

At the scheduled necropsy on day 29 of gestation, females were sacrificed by intravenous injection of sodium pentobarbitone solution. Post mortem examination included gross macroscopic examination of all internal organs, with emphasis on the ovaries, uterus and foetus. The ovaries and uteri were removed and examined and the following data recorded: pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations.

The uterus of any apparently non-pregnant female was immersed in a 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live foetuses were killed by intraperitoneal injection of sodium pentobarbitone solution. Individual foetal and placental weights were recorded and foetuses were examined externally and sexed by internal gonadal inspection.

The foetuses were stored in 10% neutral-buffered formalin.

FINDINGS:

Clinical signs and mortality:

There were no post-dosing observations or treatment-related clinical observations.

One animal of the 1000 mg/kg/day dose group (animal no. 25) presented the clinical observation 'head tilt'. This animal was further examined by a veterinary surgeon and no infection or other health problems were found. This finding was not considered to be treatment-related.

There were no treatment-related deaths during the study.

One animal was found dead on day 5 of gestation, necropsy revealed findings fairly common in this strain of rabbit. The death of this animal resulted in the control group having 6 animals, rather than 7.

Food consumption, body weight and body weight gain:

Mean body weight, mean body weight gain and mean gravid uterus weight were unaffected by treatment.

A statistically significant dose-response ($p < 0.05$) in body weight loss among animals treated at 500 and 1000 mg/kg bw/day was observed on the first day of dosing. However, this was considered not to be treatment-related due to the body weight loss seen prior to treatment in these groups.

Mean food consumption at the high dose level was very slightly lower than controls throughout the study, but was only statistically significant as a dose-response on the first day of dosing ($p < 0.05$).

Sacrifice and pathology:

There were no dose-related gross necropsy findings.

Terminal necropsy on day 29 of pregnancy found a large placenta in one animal of the high dose group. In the absence of any other observations this isolated finding was not considered to be related to treatment.

The number of pregnancies was 5, 6, 7 and 6 in the control, 250, 500 and 1000 mg/kg bw/day groups, from group sizes of 6, 7, 7, and 7, respectively. The mean numbers of corpora lutea, implantations and the mean incidence of pre- and post-implantation loss showed no treatment-related adverse effects. Mean litter size was unaffected by treatment.

Foetal data:

There was no effect of treatment on sex ratio, mean litter weight or mean placental weight. In the high dose group mean foetal weight was slightly, but not significantly lower than the controls and this was considered to be a result of higher litter size at this dose level.

There was no effect of treatment on the mean incidence of external foetal variations and malformations. In the high dose group there was one malformed foetus with Spina bifida, severely malformed head structures and severely flexed forelimb wrist joints. This was an isolated finding and was not considered to be related to treatment by the study authors.

Although statistical significance was not reached, there were more foetuses with variations in the highest dose group (slightly enlarged bilateral eye bulge, upper incisor not erupted).

Table 61: Results of the preliminary developmental study in rabbit

Dose (mg/kg bw/day)	**0**	**250**	**500**	**1000**
Number of females inseminated	6	7	7	7
Mean food consumption: Day 7 (g/animal/day)*	150	126	99	95
Mean food consumption: Day 28 (g/animal/day)	140	122	133	118
Non-pregnant	1	1	0	1
Pregnant does dead or moribund	0	0	0	0
Abortion/ premature delivery	0	0	0	0
Number of litters for evaluation	5	6	7	6
Maternal bodyweight: Day 7 (kg)	3.54	3.56	3.53	3.50
Maternal bodyweight: Day 29 (kg)	3.92	3.97	3.87	3.85
Number of implantations/doe	7.0	8.7	6.3	10.2
Mean litter size (live foetuses)	6.6	7.2	5.9	9.0
Number of foetuses examined Number of litters examined	33 5	43 6	41 7	54 6
Number of foetuses showing malformations Mean % of foetuses examined Number of litters affected	0 0.0 0	0 0.0 0	0 0.0 0	1 1.3 1
Number of foetuses showing variations Mean % of foetuses examined Number of litters affected	0 0.0 0	0 0.0 0	1 1.3 1	5 8.3 3

*: Significant dose response test ($p < 0.05$).

CONCLUSION:

Small groups of presumed-pregnant female rabbits were administered S-2200 TG at doses of 0, 250, 500 or 1000 mg/kg bw/day as a suspension in 0.5% w/v aqueous methylcellulose solution from days 7 to 29 of pregnancy, sacrificed shortly before term, and uterine contents examined for indications of effects on development.

Dose-related reduced food consumption was seen at the top dose level (1000 mg/kg bw/day), which was statistically significant as a dose-response only on the first day of dosing. Otherwise, no effect on progression of pregnancy or on development of the foetus was detected.

One foetus in the high dose group showed Spina bifida, severely malformed head structures and *severely flexed forelimb wrist joints. This was an isolated finding and was not considered to be* treatment-related.

Based on these results, both the maternal and the developmental no-observed-adverse-effect-level (NOAEL) were set at 1000 mg/kg bw/day.

On the basis of this study, a limit dose level of 1000 mg/kg bw/day was recommended as an appropriate high dose level for a further study.

Reference:	S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rabbit. Covance Laboratories Ltd, Study No. 8202046
Author(s), year:	Rhodes, J.; 2012b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0052
Guideline(s):	Japanese MAFF 12 Nousan 8147, EPA OPPTS 870.3700, OECD 414
GLP:	Yes (lab certified by National Authority)
Deviations:	None
Validity:	Yes

MATERIAL AND METHODS:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G
Purity:	93.4%
Expiry date:	21 November 2011 (after completion of treatment)
Vehicle:	0.5% w/v aqueous methylcellulose
Test animals:	
Species:	Rabbit (time mated)
Strain:	New Zealand White (Hsd:IfNZW)
Age at receipt:	4-5 months at time of mating, received time mated
Weight:	2.6 – 4.05 kg at time of mating
Source:	Harlan (UK) Ltd, Loughborough, UK
Diet:	Harlan Teklad 2930C Rabbit Diet, Pelleted, (Harlan Teklad, Madison, USA) *ad libitum*
Acclimation period:	Time mated rabbits received day 1 or 2 of gestation (day of mating = day 0). First dose administered on day 7 of gestation
Housing:	Individually

The purpose of the study was to detect adverse effects of mandestrobin (S-2200 TG) on pregnant rabbits and on the development of embryos and the foetuses consequent to exposure of the females to the test substance (0, 100, 300, and 1000 mg/kg bw/d).

Animal assignment and treatment:

On the day of arrival the time-mated and presumed pregnant animals were assigned to treatment groups each of 24 animals using a randomisation procedure based on day of gestation and body weight.

The day of insemination was designated day 0 of gestation. Rabbits were dosed once each day during days 7 to 28 of gestation. The dose volume was 5 mL/kg.

The high dose level (1000 mg/kg/day) was selected on the basis of the results of a range-finding study (Covance study number 8202040).

Table 62: Study design of the developmental study in rabbits

Group	Dose level (mg/kg bw/day)	No. Females
1	0	24
2 (low)	100	24
3 (mid)	300	24
4 (high)	1000	24

Diet preparation, analysis and administration:

Formulations of the test article in the vehicle were prepared weekly and dispensed as daily aliquots. Stability analyses were performed at the test laboratory where concentrations of 2 and 250 mg/mL were found to be stable for up to 17 days when stored at room temperature. Homogeneity was determined from formulations prepared for the first and last day of dosing.

Animals were administered the test material by gavage, once per day on days 7 – 28 of pregnancy. Control animals received 0.5% aqueous methyl cellulose.

Statistics:

Body weight gains, day 29 corrected body weights, food consumption and litter weights were analysed using one-way analysis of variance (ANOVA). Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A linear contrast was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($p < 0.01$), the data were analysed using the same methods after applying a log-transformation.

Foetal weights, placental weights, the percentage of male foetuses and the numbers of corpora lutea, implantations and foetuses per female were analysed using nonparametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

Gravid uterus weights were analysed using Analysis of Covariance (ANCOVA) and Dunnett's test, using the corrected body weight on day 29 as covariate. This analysis depends on the assumption that the relationship between the organ weight and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed. Where this showed evidence of heterogeneity ($p < 0.01$), the organ was analysed using one-way ANOVA on absolute organ weights and organ to necropsy body weight ratios.

The proportions of females affected by pre- and post-implantation loss, by early and late intrauterine deaths, the proportion with one or more dead foetuses and the number of litters affected by variations and malformations were analysed using the CochranArmitage test for dose-response and Fisher's exact test for pairwise comparisons. The tests were interpreted with one-sided risk for increased incidence with increasing dose. A significant trend ($p < 0.05$) was only reported where none of the pairwise comparisons was significant.

The percentage of foetuses in each litter affected by variations and malformations were also analysed using non-parametric methods, as previously described.

Clinical observations:

All animals were examined at least once daily for signs of ill health or overt toxicity. Any abnormalities of appearance or behaviour or other signs of reaction to treatment or ill health were recorded. An individual record was maintained of the clinical condition of each animal on each day from days 3 to 29 of gestation. On occasion, minor observations were recorded for the cage tray liner, and these were not reported.

In addition, the animals were observed immediately upon return to the home cage after dosing and at approximately 0.5 and 1 hour post dose.

Food consumption and body weight:

Individual food consumption was recorded daily from day 3 to 29 of gestation. The body weight of each female was recorded on days 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29 of gestation.

Sacrifice and pathology:

All surviving females were sacrificed on gestation day 29, by i.v. injection of sodium pentobarbitone.

Once death had been confirmed, major blood vessels were severed to exsanguinate the animal. Animals were killed in cage order and examined macroscopically. Gross lesions were retained in 10% neutral buffered formalin.

The ovaries and uteri were removed and examined and the following data recorded:

pregnancy status, gravid uterus weight, number of corpora lutea, number and intrauterine position of implantations (subdivided into: live foetuses, early intrauterine deaths, late intrauterine deaths, dead foetuses)

Early intrauterine deaths were classified as those which showed decidual or placental tissue only. The uterus of any apparently non-pregnant female was immersed in 10% ammonium sulphide solution to reveal any evidence of implantation.

Foetal examination:

Live foetuses were killed by an intraperitoneal injection of sodium pentobarbitone solution. Individual foetal and placental weights were recorded and foetuses were examined externally.

Approximately one half of the foetuses in each litter (selected by systematic sampling) were decapitated by a cut through the neck at the base of the skull. The heads were placed in Bouin's solution, for fixation and partial decalcification. Serial sections were examined and were preserved in10% neutral buffered formalin (NBF).

The hearts of approximately one half of the foetuses in each litter (selected by systematic sampling) were placed in Bouin's solution, for fixation. Several coronal slices of each heart were made to reveal the internal structure. These heart sections were examined and then preserved in NBF.

All foetuses were dissected, sexed and the viscera examined. They were then eviscerated and the carcasses fixed in 70% industrial methylated spirits.

Each carcass was processed to stain the ossified skeleton by the Alizarin technique and the skeletons were examined, including a basic evaluation of cartilage. They were preserved in glycerol/propylene glycol.

Foetal abnormalities were classified as malformations (rare and/or potentially lethal) and variations (commonly occurring non-lethal abnormalities).

FINDINGS:

Clinical signs and mortality:

There were no unscheduled deaths during the study.

There were no post-dose observations and no significant findings seen at necropsy.

Clinical observations were generally unremarkable and showed no dose-related trend. Animal number 43 receiving 100 mg/kg/day was recorded as being thin from Days 7 to 15 of gestation.

Animal number 62, receiving 300 mg/kg/day aborted its pregnancy on Day 20 of gestation. At necropsy, this animal was found to have dark foci on its lung. This abortion was not considered to be an effect of treatment due to it being in one animal only and not seen at the high dose level of 1000 mg/kg bw/day.

Food consumption, body weight and body weight gain:

Group mean body weight gain during the study was highly variable with no marked adverse effect seen. During the dose period mean body weight gain was slightly reduced compared to controls at all dose levels, although not in a dose-proportional manner and this showed apparent statistical significance as a dose-response over Days 7 to 8 and Days 17 to 19 of gestation (both $P<0.05$). Mean body weight gain after dosing ceased was slightly lower than controls at 300 and 1000 mg/kg/day, and this was statistically significant (dose response, $p < 0.05$).

Gravid uterus weight adjusted for body weight, was unaffected by treatment.

Over the dose period, mean food intake at all dose levels was slightly lower than control and this was statistically significant at 300 and 1000 mg/kg bw/day ($p < 0.01$, $p < 0.05$, respectively). However, in the pre-dose period, food intake at 1000 mg/kg/day was also slightly lower than controls, and the difference was comparable with that during the treatment phase. Mean food intake after dosing ceased was lower than controls at all dose levels, statistically significant at 300

and 1000 mg/kg bw/day ($p < 0.05$, $p < 0.01$ respectively). These slight differences from control are considered unlikely to be indicative of significant systemic toxicity to the pregnant rabbit.

Caesarean data:

Uterine/implantation data:

The number of pregnancies was 23, 23, 22 and 18, and there were 23, 23, 21 and 17 surviving females with litters on Day 29 of gestation in the control, low, intermediate and high dose groups respectively. The distribution of non-pregnant animals in the high dose group (6) was considered a chance event by the authors, and unrelated to treatment, as any increase in very early post-implantation losses would have been seen as an increase in this parameter in the mean data. Implantation in rabbits occurs at days 7-7.5 after insemination, at the beginning of the dosing period (Developmental and Reproductive Toxicology- A Practical Approach, Second Edition edited by Ronald D Hood, Taylor and Francis 2006, p163).

One animal receiving 1000 mg/kg bw/day (number 93) had total embryo-foetal loss; this isolated incidence was not considered to be an effect of treatment by the study authors. The mean numbers of corpora lutea, implantations and the mean incidence of pre- and post-implantation loss showed no effect of treatment. The number of dams with post-implantation loss at 100 mg/kg bw/day showed an apparent statistical significance, however all data were within expected ranges and this was not considered to be biologically significant.

Mean litter size was unaffected by treatment.

Table 63: Uterine/ implantation data

Dose (mg/kg bw/day)	**0**	**100**	**300**	**1000***	**HCD mean (range)****
Mean number of corpora lutea	10.3	10.1	10.0	9.7 (9.4)	10.4 (10.0 - 11.9)
Mean number of implantations	8.9	9.4	8.8	8.8 (8.4)	8.6 (7.6 - 9.4)
Pre-implantation loss (%)	15.0	7.7	12.9	9.1 (13.0)	17.3 (9.5 – 27.6)
Intrauterine deaths early	0.6	0.9	0.8	0.9 (0.9)	0.6 (0.4 – 0.8)
Intrauterine deaths late	0.2	0.3	0.2	0.3 (0.3)	0.2 (0.1 – 0.3)
Dead foetuses	0.0	0.0	0.0	0.1 (0.1)	0.0 (0.0)
Post implantation loss (%)	8.7	13.6	11.0	13.3 (18.1)	9.1 (7.1 – 14.2)
Live foetuses	8.0	8.2	7.8	7.5 (7.1)	7.8 (6.9 – 8.7)

*The animal with total embryo-foetal loss was excluded from the analysis; values in brackets contain this doe in the analysis
**Historical control data was derived from 6 embryo foetal studies performed with New Zealand White rabbits at Covance from April 2004

Foetal data:

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean foetal weight.

Malformations were noted in eight foetuses from six litters in the control group, six foetuses from four litters in the group receiving 100 mg/kg bw/day, nine foetuses from seven litters in the group receiving 300 mg/kg bw/day and three foetuses from three litters in the group receiving 1000 mg/kg bw/day.

Table 64: Necropsy findings in the developmental study in rabbit

Dose (mg/kg bw/day)	**0**	**100**	**300**	**1000**	**HCD***
Number of females inseminated	24	24	24	24	-
Non-pregnant	1	1	2	6	-
Pregnant (%)	23 (95.8)	23 (95.8)	22 (91.7)	18 (75.0)	(83.3 – 100)
Accidental death	0	0	0	0	-
Abortion/ premature delivery	0	0	1	0	-
Total litter resorptions	0	0	0	1	-
Number of litters for evaluation	23	23	21	17	-
Maternal body weight (kg), day 7	3.32	3.28	3.31	3.44	-
day 29 (surviving does)	3.83	3.72	3.70	3.87	-
Number of implantations/doe	8.9	9.4	8.8	8.8	-
Resorption & foetal death (%)	8.7	13.6	11.0	13.3	-
Mean litter size (live foetuses)	8.0	8.2	7.8	7.5	6.9 – 8.7
Mean foetal weight (g): Male	44.4	43.9	43.0	45.9	-
Female	45.1	43.4	41.7	45.4	-
Sex ratio (% of male)	49.1	50.3	57.6	50.0	45.1 – 50.7
Foetuses with external or visceral malformations, n (%)	7 (3.3)	4 (1.5)	2 (1.2)	2 (1.7)	(2.6)
Litter incidence	6/23	3/23	2/21	2/17	-
Foetuses with external or visceral Variations, n (%)	98 (55.3)	85 (46.0)	80 (49.4)	54 (43.0)	(48.7)
Litter incidence	22/23	23/23	21/21	15/17	-
Foetuses with skeletal malformation n (%)	3 (1.5)	4 (1.7)	7 (4.9)	2 (1.6)	(2.7)
Litter incidence	2/23	3/23	6/21	2/17	-
Foetuses with skeletal variations n (%)	148 (79.8)	142 (75.0)	114 (70.8)	110 (85.8)	(73.9)
Litter incidence	22/23	23/23	21/21	17/17	-

Total number of foetuses with malformations, n (%)	8 (4.3)	6 (3.2)	9 (5.5)	3 (2.3)	-
Litter incidence	6/23	4/23	7/21	3/17	-
* Historical control data was derived from 6 embryo foetal studies performed with New Zealand White rabbits at Covance from April 2004					

Table 65: Detailed representation of visceral malformations (including historical control data)

External or visceral malformations	**Numer of foetuses affected (mean % of foetuses)**				**HCD (%)[a]**
Dose (mg/kg bw/day)	**0**	**100**	**300**	**1000**	
Abdomen					
stomach, displaced - cranial to liver	1 (0.5)	1 (0.3)		1 (0.8)	0 (0.0)
abdominal viscera, situs inversus		1 (0.3)			0 (0.0)
kidney, absent	1 (0.4)	1 (0.3)			0 (0.0)
kidney, displaced - cranial		1 (0.3)			0 (0.0)
spleen, absent		1 (0.3)		1 (0.8)	0 (0.0)
ureter, absent	1 (0.4)	1 (0.3)			0 (0.0)
Blood vessel(s)					
aortic arch, dilated		2 (0.9)		1 (0.8)	0-2 (0.0-1.2)
aortic arch, retro-oesophageal	1 (0.5)				0 (0.0)
aortic arch, right-sided	1 (0.5)	1 (0.3)		1 (0.8)	0 (0.0)
ductus arteriosus, absent	1 (0.5)	1 (0.4)			0-1 (0.0-0.6)
pulmonary artery, arising from aorta	1 (0.5)			1 (0.8)	0 (0.0)
pulmonary trunk, narrowed		2 (0.8)			0-2 (0.0-1.2)
pulmonary trunk, reduced to non-patent tissue strand		1 (0.4)		1 (0.8)	0 (0.0)
subclavian artery, arising from descending aorta	5 (2.2)	2 (0.8)	2 (1.2)	1 (0.8)	0-6 (0.5-3.4)
aortic arch, branched				1 (0.8)	0 (0.0)
ascending aorta, dilated				1 (0.8)	0-1 (0.0-0.6)
branches from left subclavian artery, origin displaced		1 (0.3)			0 (0.0)
common carotid artery, arising from pulmonary trunk	1 (0.5)				0 (0.0)
descending aorta, right-sided				1 (0.8)	0 (0.0)
ductus arteriosus, narrowed		1 (0.3)			0-2 (0.0-1.2)
ductus arteriosus, pathway abnormal				1 (0.8)	0 (0.0)
pulmonary trunk, lumen not patent		1 (0.3)			0 (0.0)
subclavian artery, retro-oesophageal	5 (2.2)	3 (1.1)	2 (1.2)		0-1 (0.0-0.6)
Eye(s)					
reduced in size		1 (0.3)			0 (0.0)
Head					
frontal/parietal region, opening in skull - brain extruded		1 (0.3)			0-1 (0.0-0.6)
parietal region, flattened				1 (0.8)	0 (0.0)
parietal/occipital region, flattened		1 (0.3)			0 (0.0)
frontal region, flattened		1 (0.3)			0 (0.0)
orbital region, absent		1 (0.3)			0 (0.0)
orbital region, reduced in size		1 (0.3)			0 (0.0)
pinna, reduced in size	1 (0.4)				0 (0.0)
Head - Buccal cavity					
palate, cleft		1 (0.3)			0-3 (0.0-1.7)

Heart					
right atrium, enlarged	1 (0.5)				0 (0.0)
right ventricle, outlet to aorta		1 (0.4)			0-1 (0.0-0.6)
ventricles, thickened		2 (0.9)			
rotated - apex directed ventrally	1 (0.4)				0 (0.0)
interventricular septum, incomplete	1 (0.5)	2 (0.8)		1 (0.8)	0-2 (0.0-1.4)
left ventricle, reduced in size		1 (0.3)			0 (0.0)
right atrium, thickened	1 (0.5)				0 (0.0)
ventricles, misshapen		1 (0.4)			0 (0.0)
interventricular septum, thickened	1 (0.5)				0 (0.0)
left ventricle, no arterial outlets	1 (0.5)				0-1 (0.0-0.6)
right ventricle, reduced in size	1 (0.5)	1 (0.4)			0 (0.0)
left atrium, absent	1 (0.5)				0 (0.0)
Limb(s), hind					
knee joint, flexed				1 (0.8)	0 (0.0)
Neck/Thorax					
lung, lobulation abnormal		1 (0.3)			0 (0.0)
lung, reduced in size		1 (0.3)			0-1 (0.0-0.6)
Trunk					
abdomen, opening in body wall involving umbilicus	1 (0.4)				0 (0.0)
back, opening in skin - meninges protruding	1 (0.6)				0 (0.0)
tail, shortened				1 (0.8)	0-1 (0.0-0.6)
umbilical opening, displaced - cranial	1 (0.4)				0 (0.0)
Vertebral column					
open - spinal cord exposed, lumbar region				1 (0.8)	0 (0.0)

a: Values are calculated by the following formula: (number of foetuses affected/ number of foetuses examined) x100

Table 66: Detailed representation of skeletal malformations (including historical control data)

External or visceral malformations	**Numer of foetuses affected (mean % of foetuses)**				**HCD (%)[a]**
Dose (mg/kg bw/day)	**0**	**100**	**300**	**1000**	
Pectoral girdle					
clavicle, interrupted		1 (0.3)			0 (0.0)
Rib(s)					
11, absent			1 (0.7)		0 (0.0)
4, shortened		1 (0.3)			0 (0.0)
indeterminable, one absent		1 (0.3)			0 (0.0)
rib(s), abnormal attachment		1 (0.3)	1 (0.7)		0 (0.0)
rib(s), branched			2 (1.3)		0-1 (0.0-1.5)
rib(s), fused - proximal		1 (0.3)			0-2 (0.0-1.1)
Vertebral - Caudal vertebra(e)					
proximal, splayed	1 (0.6)			1 (0.8)	0 (0.0)
mid, disorganised				1 (0.8)	0 (0.0)
mid, misaligned				1 (0.8)	0 (0.0)
Vertebral - Cervical arch(es)					
1, reduced in size		1 (0.3)			0 (0.0)
Vertebral - Cervical centrum/a					
3, hemicentric - right side present		1 (0.3)			0 (0.0)
7, hemicentric - left side present		1 (0.3)			0 (0.0)
odontoid process, absent		1 (0.3)			0 (0.0)
ventral arch of vertebra 1, misshapen		1 (0.3)			0 (0.0)
Vertebral - Lumbar arch(es)					

5/6, additional right arch between			1 (1.0)		0 (0.0)
lumbar arch(es), splayed				1 (0.8)	0 (0.0)
Vertebral - Lumbar centrum/a					
6, fused to centrum of adjacent vertebra		1 (0.3)			0 (0.0)
6/7, fused			1 (1.0)		0 (0.0)
Vertebral - Lumbar vertebra(e)					
6, hemivertebra - left side present		1 (0.3)			0-1 (0.0-0.7)
lumbar vertebra(e), misaligned		1 (0.3)			0-1 (0.0-0.7)
Vertebral - Sacral arch(es)					
sacral arch(es), splayed	1 (0.6)			1 (0.8)	0 (0.0)
Vertebral - Thoracic arch(es)					
7/8, fused		1 (0.3)			0.0
8, reduced in size		1 (0.3)			0-1 (0.0-0.6)
last, splayed		1 (0.3)			0 (0.0)
Vertebral - Thoracic centrum/a					
11, fused to centrum of adjacent vertebra			1 (0.7)		0 (0.0)
4, absent		1 (0.3)			0 (0.0)
7/8, fused		1 (0.6)			0 (0.0)
8, hemicentric - left side present		1 (0.3)			0 (0.0)
1, hemicentric - left side present		1 (0.3)			0 (0.0)
Vertebral - Thoracic vertebra(e)					
11, hemivertebra - left side present			1 (0.7)		0-1 (0.0-0.5)
indeterminable, one absent		1 (0.3)			0 (0.0)
Vertebral column					
lateral curvature - lumbar region		1 (0.3)			0-1 (0.0-0.7)
lateral curvature - thoracic region		1 (0.3)			0-1 (0.0-0.5)

a: Values are calculated by the following formula: (number of foetuses affected/ number of foetuses examined) x100

Overall, there was no effect of treatment on the incidence of foetal variations and malformations, which were all within expected ranges for this strain of rabbit.

CONCLUSION:

In a developmental toxicity study, groups of 24 presumed-pregnant female rabbits were administered mandestrobin (S-2200 TG) at doses of 0, 100, 300 or 1000 mg/kg bw/day by gavage from days 7 to 28 of pregnancy.

There were no significant adverse post-dosing observations, and no significant treatment-related clinical signs or necropsy findings. Mean food consumption and mean body weight gain at all dose levels were variable and generally were slightly reduced compared to controls during the dosing period, and upto necropsy. However, these slight differences were very small in magnitude and are considered unlikely to represent significant systemic toxicity.

One animal receiving 300 mg/kg bw/day aborted its pregnancy on day 20 of gestation with no significant clinical signs or necropsy findings, and was not considered to be treatment-related. There were no other unscheduled deaths during the study.

Mean gravid uterus weight adjusted for body weight, was unaffected by treatment. There was no effect of treatment on mean uterine/implantation data.

Sex ratio, mean litter weight, mean placental weight and mean foetal weight were all unaffected by treatment and there was no effect of treatment on the incidences of foetal variations or malformations.

In conclusion, administration of S-2200 TG by oral gavage to pregnant rabbits elicited no systemic toxicity to maternal female rabbits. There was no evidence of embryotoxicity or developmental effects at any dose level tested. Under the conditions of this study, both the maternal and the embryo-foetal no-observed-adverse-effect-level (NOAEL) were set at 1000 mg/kg bw/day.

4.11.2.2 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.11.3 Other relevant information

No other relevant information available.

4.11.4 Summary and discussion of reproductive toxicity

Mandestrobin was examined in a two-generation reproductive toxicity study in the rat and in teratogenicity studies in the rat and rabbit.

In the 2-generation reproductive toxicity study (doses of 1000, 3000, and 10000 ppm via diet), mandestrobin showed no evidence of an effect on fertility or reproductive function, resulting in a NOAEL for the reproductive effects at the highest dose level tested in this study of 10000 ppm (559 mg/kg bw/day).

Regarding the general toxicological effects on parental animals, suppressed body weight gain and reduced food consumption were noted in males and in females in the 10000 ppm group in both F0 and F1 generations.

Pathological examination revealed treatment-related changes in the liver of both sexes. At necropsy, dark brownish change and enlargement of the liver were noted in F0 and F1 females in the 10000 ppm group. Liver weights increased in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the histopathological examination the following findings were observed: Brown pigment in the bile duct/periportal area (in F0 animals at 10000 ppm and in F1 animals at ≥ 3000 ppm), focal periductular inflammatory cell infiltration (in F0 males and females and F1 males at 10000 ppm and in F1 females at ≥ 3000 ppm), and brown pigment deposition in the perilobular hepatocyte and proliferation of the bile duct (in F0 and F1 females in the 10000 ppm group). Diffuse hypertrophy of hepatocytes was also observed in males at ≥ 3000 ppm and in females at ≥ 1000 ppm in both generations. In the 1000 ppm group, hepatocellular hypertrophy and increased liver weights were observed in F0 and F1 females.

Furthermore, increases in the thyroid weights were observed in F0 males at ≥ 3000 ppm and in F1 males at 10000 ppm, and hypertrophy of the follicular cell of the thyroid was observed in some F0 males in the 10000 ppm group, which was considered to be secondary to the increased in hormonal turnover and the changes in the liver.

Treatment-related hypertrophy of cortical cells in the fascicular zone was observed in the adrenals in some F1 females in the 10000 ppm group. In addition, decreases were observed in ovary weights in F0 and F1 females and uterus weights in F0 females in the 10000 ppm group, however, in absence of any histopathological changes.

Furthermore, greenish change in the cortex of the kidney was observed in some F0 females at necropsy in the 10000 ppm group, with increased organ weights but without any histopathological changes. Therefore, the changes in the kidney observed in F0 females were considered to be of no toxicological significance.

Because of the liver effects (weight increase, diffuse hepatocellular hypertrophy) observed at the lowest dose level in parental animals, no parental NOAEL was set for this study. The lowest dose is considered a LOAEL.

Regarding the effects on offspring, postnatal body weight gain was suppressed in both sexes of F1 and F2 offspring in the 10000 ppm group, and was probably due to undernourishment of their dams.

In addition, lower spleen weights at weaning were noted in F1 males in the 3000 ppm group, in which no change was found in postnatal body weight gain, and in all F1 and F2 animals at 10000 ppm. The lower spleen weights of F1 animals at weaning completely recovered to the control level at adulthood in both sexes, even in the 10000 ppm group, suggesting a transient retardation in growth. Moreover, both the absolute and relative spleen weights of F1 and F2 animals were within the historical control range in the test facility, indicating slight changes.

A slight delay in sexual maturation was found in both sexes (mean difference from the control group: 1.5 days for vaginal opening in F1 females and 1.6 days for preputial separation in F1 males) in the 10000 ppm group, which was considered to be related to the growth retardation.

Under the conditions of this study, the NOAEL for effects on offspring is considered to be 1000 ppm (56 mg/kg bw/d).

In a gavage developmental toxicity study, administration of 0, 100, 300, and 1000 mg/kg bw/day from Days 6 to 19 of gestation to pregnant rats elicited no treatment-related adverse effects up to the highest dose level tested. Therefore, both the maternal and the foetal NOAEL were set at 1000 mg/kg bw/day.

Administration of mandestrobin by oral gavage to pregnant rabbits at doses of 0, 100, 300 or 1000 mg/kg bw/day from Days 7 to 28 of pregnancy elicited no systemic toxicity to maternal female rabbits. There was no evidence of embryotoxicity or developmental effects at any dose level tested. Under the conditions of this study, both the maternal and the foetal NOAEL were set at 1000 mg/kg bw/day.

4.11.5 Comparison with criteria

In the developmental toxicity studies in rat and rabbit, no treatment-related adverse effects were observed in dams or in foetuses, whereas some adverse effects were observed in the 2-generation reproductive toxicity study in the rat.

Adverse effects to be discussed with regard to sexual function and fertility are decreases in ovary and uterus weights as well as a slight delay in sexual maturation: Decreases were observed in ovary weights in F0 and F1 females and uterus weights in F0 females in the 10000 ppm group, however, in absence of any histopathological changes and in absence of of an effect on fertility or reproductive function. A slight delay in sexual maturation was found in both sexes (mean difference from the control group: 1.5 days for vaginal opening in F1 females and 1.6 days for preputial separation in F1 males) in the 10000 ppm group, which was considered to be related to the growth retardation.

Adverse effects to be discussed with regard to development of the offspring are reduced postnatal body weight gain and lower spleen weight at weaning: Postnatal body weight gain was suppressed in both sexes of F1 and F2 offspring in the 10000 ppm group, probably due to undernourishment of their dams. In addition, lower absolute and relative spleen weights at weaning (within the historical control range) were noted in F1 males in the 3000 ppm group, in which no change was found in postnatal body weight gain, and in all F1 and F2 animals at 10000 ppm. The lower spleen weights of F1 animals at weaning completely recovered to the control level at adulthood in both sexes, even in the 10000 ppm group, suggesting a transient retardation in growth.

Taken together, no classification and labelling is triggered for mandestrobin based on the results of the 2-generation reproduction toxicity study in the rat and of two developmental toxicity studies in rat and rabbit.

4.11.6 Conclusions on classification and labelling

There is no evidence of adverse effects on either fertility and sexual function (f/F) or on development (d/D) caused by mandestrobin. Therefore, no classification and labelling is proposed.

4.12 Other effects

4.12.1 Non-human information

4.12.1.1 Neurotoxicity

Table 67: Summary table of relevant neurotoxicity studies

Method	Dose range / NOAEL / Effects	Remarks	Reference
Oral (Gavage) Dose Range-Finding Acute Neurotoxicity Study in Wistar Rats (No appropriate guideline existing for this study)	0, 300, 1000, 2000 mg/kg bw NOAEL: 2000 mg/kg bw	Crl:WI(Han) rats Purity: 93.4%	Herberth, M.T.; 2011a

	Effects: No effect at highest dose tested		
Oral (Gavage) Acute Neurotoxicity Study in Wistar Rats (OECD 424)	0, 500, 1000, 2000 mg/kg bw NOAEL: 1000 mg/kg bw Effects: Decreased overall locomotor activity (total and/or ambulatory counts) in males and females at 2000 mg/kg bw	Crl:WI(Han) rats Purity: 93.4%	Herberth, M.T.; 2011b
90-day oral dietary neurotoxicity study in rats (OECD 424)	0, 1500, 5000, 15000 ppm equivalent to: 0, 99, 338, 1024 mg/kg bw/d in males and 0, 122, 415, 1223 mg/kg bw/d in females Neurotoxicity NOAEL: 1024 mg/kg bw/d Systemic toxicity NOAEL: 338 mg/kg bw/d ♂ 1223 mg/kg bw/d ♀ Effects: No neurotoxicity at highest dose tested ↓ body weight, body weight gain and food consumption in males at 1024 mg/kg bw/d	Crl:WI(Han) rats Purity: 93.4%	Herberth, M.T.; 2012

The potential for mandestrobin to cause neurotoxicity was thoroughly assessed in acute and subchronic neurotoxicity studies. It is noted that these studies were conducted for other regulatory authorities, since there is no toxicity that triggers these studies as a requirement under EU regulations.

In a range-finding assay designed to identify the time of peak effect after an acute oral limit dose (2000 mg/kg bw), no reaction was seen, so no peak effect was evident. In the subsequent definitive acute neurotoxicity study, investigations intended for the "time of peak effect" were therefore conducted at the latest timepoint compatible with guideline, i.e. 8 hours post-dose.

The only treatment-related finding in the definitive acute neurotoxicity study was a decrease in mean locomotor activity, seen at the top dose (2000 mg/kg bw) at 8 hours post-dose. In the absence of any other specific neurological findings, this is probably attributable to transient systemic toxicity rather than typical neurotoxicity. The NOAEL, set on a highly precautionary basis assuming a decrease in locomotor activity in absence of any other neurological change might be meaningful, was 1000 mg/kg bw.

In a 90-day subchronic neurotoxicity study, at doses of up to 15000 ppm (equivalent to 1024 and 1223 mg/kg bw/d in males and females, respectively), no evidence of specific neurotoxicity was found. The NOAEL for specific neurotoxicity was therefore approximately 1024 mg/kg bw/day. On the basis of body weight gain retardation at the top dose in males only, the NOAEL for systemic toxicity was 338 mg/kg bw/day.

4.12.1.2 Immunotoxicity

Table 68: Summary table of relevant immunotoxicity studies

Method	Dose range / NOAEL / Effects	Remarks	Reference
Rat, 28-days (oral), range finding study for immuntox and neurotox studies (in general accordance with US EPA OPPTS 870.7800)	0, 1500, 5000, 15000 ppm Equivalent to ♂: 0, 132, 430, 1200 mg/kg bw/day ♀: 0, 135, 436, 1340 mg/kg bw/day NOAEL: ♂ 1200 mg/kg bw/day ♀ 1340 mg/kg bw/day Effects: No treatment related effects observed at the highest dose level tested	Crl:WI(Han) rats Purity: 93.4%	Hosako, H., 2011a
Rat, 28-days (oral), immunotox study (US EPA OPPTS 870.7800)	0, 1500, 5000, 15000 ppm Equivalent to ♀: 0, 147, 471, 1419 mg/kg bw/day NOAEL: 1419 mg/kg bw/day Effects: No treatment related effects observed at the highest dose level tested	Crl:WI(Han) rats, females only Purity: 93.4%	Hosako, H., 2011b

In a 28-day dietary dose range finding study in Wistar Han rats, no mandestrobin-related effects were observed on the AFC response in a splenic antibody-forming cell (AFC) assay. Therefore, the no-observed-effect-level (NOEL) for the humoral immune response in the T-cell dependent antibody response (TDAR) groups was considered to be 15000 ppm, equivalent to 1340 mg/kg of body weight/day, which is exceeding the limit dose for such studies. No adverse effect was observed for the general toxicity parameters evaluated. A dose level of 15000 ppm was concluded to be appropriate for subsequent definitive immunotoxicity testing.

In the oral immunotoxicity study, mandestrobin administered in the diet *ad libitum* for 28 consecutive days to 3 groups of female Crl:WI(HAN) rats at dosage levels of 1500, 5000, and 15000 ppm did not suppress the AFC response to the T cell-dependent antigen sheep red blood cells. Therefore, the no-observed-adverse-effect level (NOAEL) for the functional humoral immune response was considered to be 15000 ppm (equivalent to 1419 mg/kg/day), the highest dose level tested.

4.12.1.3 Specific investigations: other studies

Table 69: Summary table of relevant *in vivo* and *in vitro* mechanistic studies and two detailed position papers dealing with (i) liver and thyroid effects and (ii) ovary issues

Study / Method	Results	Remarks	Reference
Short-Term Study for Mode of Action Analysis for Rat	Mandestrobin causes increased liver weight with diffuse, hepatocellular	Rats (Crlj:WI)	*Asano H.,*

Liver and Thyroid Findings by S-2200TG –Dose Response, Time-Course and Reversibility (no guideline, mechanistic study)	hypertrophy, proliferation of liver smooth endoplasmic reticulum, increased liver CYP2B and T4-UGT activity, and transient increase of replicative DNA synthesis in a dose dependent and reversible manner. In the thyroid, mandestrobin causes slight hypertrophy, slight decreases of serum T4 levels and slight increases in TSH in both genders after 14 days. These findings were reversible.	Purity: 93.4%	*2012e*
Short-Term Study for Mode of Action Analysis for Mouse Liver Findings by S-2200TG (no guideline, mechanistic study)	7 days treatment with mandestrobin caused slight increases of liver weight, and induction of CYP2B activity.	Mice (Crlj:CD1(ICR)), males only Purity: 93.4%	*Yamada T., 2012b*
In vitro Steroidogenesis Assay of S-2200TG in H295R Cells (OECD 456)	No influence of mandestrobin on testosterone and estradiol production was observed	Human adrenocortical NCI-H295R cell line Purity: 93.4%	*Kubo H., 2012*
Estrogen Receptor alpha and Human Androgen Receptor Using *in vitro* Reporter Gene Assays (OECD 455)	Mandestrobin and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-S-2200-NHM) did not show agonistic or antagonistic effects on hERα and hAR –induced transcriptional activation	hERα-HeLa-9903 and hAR-HeLa 4-11 cell lines Purity: 93.4%	*Suzuki N., 2012*
The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action	Mode of Action analysis for mandestrobin shows a phenobarbital-like mechanism	Position paper	*Yamada T., 2012a*
Interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study	The increased incidence of benign ovary sex-cord stromal tumour observed in females in the 2-year rat study is not considered to be a carcinogenic effect of mandestrobin and, therefore, does not trigger classification for carcinogenicity.	Position paper	*Yamada., T., Miyata, K.; 2012*
Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study		Update of position paper	*Yamada., T.; 2013*

Liver and thyroid changes

In repeat dose toxicity studies the primary target organ of mandestrobin is the liver in all species examined. The thyroid was also a target organ in the rat, but not in the mouse and dog. The primary liver finding in the rat was hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the main thyroid finding was follicular cell hypertrophy. However, no tumourigenicity was observed in rat or mouse carcinogenicity studies.

In conjunction with evidence from the literature, mode of action studies were conducted to provide evidence that mandestrobin is a hepatic enzyme inducer via at least constitutive androstane receptor (CAR) activation in rat, in a manner similar to phenobarbital (PB).

Note: The mechanistic data presented by the notifier and summarized by the rapporteur member state were considered insufficient during the pesticide peer review, as phenobarbital induced centrilobular hypertrophy, whereas the hepatocyte hypertrophy induced by mandestrobin was diffuse.

Reference:	Short-Term Study for Mode of Action Analysis for Rat Liver and Thyroid Findings by S-2200TG – Dose Response, Time-Course and Reversibility
Author(s), year:	Asano, H.; 2012e
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0067
Guideline(s):	No (mechanistic study)
GLP:	No. Conducted in a GLP-compliant facility
Deviations:	-
Validity:	Yes

The hypothesis that mandestrobin (S-2200 TG) induces hepatic metabolic enzymes via constitutive androstane receptor (CAR) activation similar to phenobarbital (PB), resulting in the liver and thyroid alterations seen in the rat studies, was tested. To evaluate the time course of alterations at 15000 ppm, 10 rats/sex/group were also fed diets containing 0 and 15000 ppm for 14 days. Data from both 7 and 14-day treatment groups were compared to determine whether enhancement or attenuation of alterations was observed. To evaluate reversibility of any findings, 10 rats/sex/group were also fed diets containing 0 and 15000 ppm S-2200 TG for 7 days followed by a 7-day recovery period. As a positive control for CAR activation, groups of rats were treated with 1000 ppm PB.

Generally the mode of action (MOA) for phenobarbital-like liver and thyroid effects can be described as follows: Phenobarbital activates nuclear receptors, particularly the constitutive androstane receptor (CAR). This receptor translocates into the nucleus and dimerizes with the retinoid-X-receptor (RXR). The dimer then binds to specific response elements, resulting in transcriptional activation of genes regulating P450 expression, particularly expression of CYP2B. In turn, CYP4A expression is associated with activation of the peroxisome proliferator-activated receptor (PPAR) (Holsapple *et al*, Toxicological Sciences 2006: 89(1), 51-56). Phenobarbital-induced liver enlargement is associated with initial transient hyperplasia, and a substantial proliferation of smooth endoplasmatic reticulum (SER), causing hepatocellular hypertrophy (Maronpot *et al*, Toxicologic Pathology 2010: 38, 776-795).

Another liver enzyme induced via the activated CAR receptor is UDP-glucoronosyltransferase (UGT). Induction of UGT leads to increased hepatic clearance of thyroxine (T4) by increased UDP glucuronosyltransferase activity towards T4 (T4-UGT). Circulating levels of T4 are monitored by the thyrotropic cells of the pituitary gland that are responsible for the synthesis of thyroid stimulating hormone (TSH). In the pituitary gland, T4 is metabolised to T3. Decreasing levels of T3 leads to stimulation of TSH synthesis and secretion. Sustained perturbation of the hypothalamic-pituitary-thyroid axis and the prolonged stimulation of the thyroid gland by TSH

can lead to the progression of thyroid follicular cells to hypertrophy, hyperplasia, and eventually neoplasia (Dellarco *et al*, Critical Reviews in Toxicology 2006: 36, 793-801).

It is generally accepted that this MOA is unlikely to be of relevance for humans, due to species differences in kinetic and dynamic factors (Holsapple *et al*, Toxicological Sciences 2006: 89(1), 51-56).

Administration of S-2200 TG resulted in increased liver weight in both genders after 7 and 14 days of administration. The changes were reversible after the 7 day recovery period. Liver weight changes caused by treatment with phenobarbital were comparable.

Whereas PB caused hepatocyte centrilobular hypertrophy in all animals in both genders, S-2200 TG caused clear increases in diffuse hepatocyte hypertrophy in both genders.

Similar stimulation of replicative DNA synthesis was caused by both substances after 7 days of substance administration, and in both cases, this response was weaker after 14 days of substance administration. Proliferation of the smooth endoplasmic reticulum was observed at 15000 ppm of S-2200 TG.

A clear induction of CYP2B activity was induced by administration of S-2200 TG and PB, which after the 7 day recovery period, was clearly decreased.

There was a weak, but statistically significant induction of CYP4A activity by PB, but not by S-2200 TG. After the 7 day recovery period, CYP4A activity was slightly increased in males and decreased in females; the biological significance of this finding is doubtful.

After 7 days, UGT activity was clearly increased in both genders after PB and S-2200 TG administration, with a clear tendency to reverse to normal after 7 days recovery.

Thyroid weight was statistically significantly increased in females after 7 days and in both genders after 14 days. This finding was associated with diffuse follicular hypertrophy. There was a tendency towards reversibility after the 7 day recovery period.

Serum T4 levels were decreased after 7 and 14 days of administration of PB and S-2200 TG, with a tendency to reverse to control levels after 7 days recovery.

Serum T3 levels were not yet decreased after 7 days of S-2200 TG treatment in both genders, 14 day treatment with S-2200 TG resulted in decreased serum T3 levels in females.

Serum TSH levels were increased in females after 7 days of treatment, and in both genders after 14 treatment days. The 7 days recovery period did lead to TSH levels had lowered.

In conclusion, the effects observed in the study are in agreement with the published MOA for phenobarbital: liver weight increase, proliferation of SER, initial stimulation of replicative DNA synthesis, specific induction of CYP2B and UGT activity, increased thyroid weight and hypertrophy and changes in serum T4, T3, and TSH levels were observed and showed clear indications of reversibility after a 7 day recovery period.

Reference:	Short-Term Study for Mode of Action Analysis for Mouse Liver Findings by S-2200TG
Author(s), year:	Yamada, T.; 2012b
Report/Doc. number:	Sumitomo Chemical Co. Ltd. Report No. ROT-0068
Guideline(s):	No (mechanistic study)
GLP:	No. Conducted in a GLP-compliant facility
Deviations:	-
Validity:	Yes

10 male mice/group were fed diets containing 0 (control) and 7000 ppm mandestrobin (S-2200 TG) for 7 days.

In the mouse, treatment with mandestrobin (S-2200 TG) resulted in increased liver weight with slight eosinophilic change/hypertrophy of hepatocytes and increased CYP2B activity. However, S-2200 TG did not enhance replicative DNA synthesis in hepatocytes during the short period of treatment.

Reference:	The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action
Author(s), year:	Yamada, T.; 2012a
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0070
Guideline(s):	Not applicable, position paper
GLP:	-
Deviations:	-
Validity:	Yes

The unchanged position paper is included into Appendix 1 of this report.

Executive Summary:

In repeat dose toxicity studies the primary target organ of mandestrobin (S-2200 TG) is the liver in all species examined. The thyroid was also a target organ in the rat, but not in the mouse and dog. The primary liver finding in the rat was hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the main thyroid finding was follicular cell hypertrophy. However, no tumourigenicity was observed in these two target organs in rat or mouse carcinogenicity studies.

In conjunction with evidence from the literature, mode of action studies were conducted to provide evidence that S-2200 TG is a hepatic enzyme inducer via at least constitutive androstane receptor (CAR) activation in the rat, in a manner similar to phenobarbital (PB). This was evidenced in the mode of action studies by induction of CYP2B activity and UDP-glucuronosyltransferase activity toward thyroxine (T4-UGT), and proliferation of smooth endoplasmic reticulum (SER). Therefore, the liver hypertrophy caused by S-2200 TG was judged to be an adaptive response via CAR mediated enzyme induction and not adverse. A similar mode of action also appears plausible in mouse and dog, and would theoretically operate in humans, as demonstrated by CYP2B induction.

At higher doses S-2200 TG induced adverse effects on the liver as evidenced by functional changes or additional pathological findings to the hypertrophy (multiple liver related blood biochemistry findings, hepatocyte vacuolation, degeneration). However, the adverse effects occurred in a dose related manner and there was a threshold at a relatively high exposure level. Most importantly, S-2200 TG did not induce liver tumours in the rat or mouse, reducing concern for the human risk assessment.

Secondly, data was obtained indicating that S-2200 TG increased T4-UGT activity, indirectly perturbing the hypothalamus-pituitary-thyroid hormone axis, and thereby inducing thyroid follicular-cell hypertrophy in rats, in a manner similar to PB, a CAR activator. The relevance of the rat thyroid abnormality to human health was assessed by using the 2008 IPCS Human Relevance Framework. The postulated mode of action (MOA) for possible induction of thyroid follicular-cell hypertrophy in rats was tested against the Bradford Hill criteria, and was found to satisfy the conditions of dose and temporal concordance, biological plausibility, coherence, strength, consistency, and specificity that fit with a well-established MOA for thyroid follicular-cell hypertrophy. Although the postulated MOA could theoretically operate in humans, marked quantitative differences in the inherent susceptibility for thyroid abnormality, especially tumour induction, to thyroid hormone imbalance in rats is not relevant to humans. Therefore, even though liver and thyroid hypertrophy were induced by S-2200 TG in experimental animals, the findings from a MOA analysis allow the conclusion that S-2200 TG does not pose a practical hazard to humans.

Ovary issues

Reference:	Interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study
Author(s), year:	Yamada., T., Miyata, K.; 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0069
Guideline(s):	Not applicable, position paper
GLP:	Not applicable, position paper
Deviations:	Not applicable, position paper
Validity:	Yes

Reference:	Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study
Author(s), year:	Yamada., T.; 2013
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0075
Guideline(s):	Not applicable, position paper
GLP:	Not applicable, position paper
Deviations:	Not applicable, position paper
Validity:	Yes

The unchanged position paper is included into Appendix 1 of this report.

Executive Summary:

Mandestrobin (S-2200 TG) was not genotoxic in a battery of *in vitro* and *in vivo* assays. The tumourigenic potential of S-2200 TG was studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by authorities.

An increased incidence of ovary sex-cord stromal tumour (SCST) was observed in female rats, exceeding the historical control range for this strain of rats. Therefore, a causal relationship between S-2200 TG administration and the ovary tumour induction was not ruled out. In the mouse study, the number of tumours in any tissue did not increase by exposure to S-2200 TG. Therefore, one tumour type (benign) in one sex (female) of one species (the rat) occurred in one study. Four and six cases of benign ovarian SCST occurred in female rats exposed to 7000 and 15000 ppm (475 and 1016 mg/kg/day) S-2200 TG, respectively. These were dose levels at which body weight gain was reduced by > 20%, indicating that the maximum tolerated dose was exceeded. It must be highlighted that also the current controls (two cases of benign ovarian SCST) exceeded the historical control range for this strain of rats.

Sex-cord stromal hyperplasia is quite common in aged Wistar rats, and the animals used in the 2-year study with S-2200 TG appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were well within historical controls for all groups, and there was no statistical difference between groups for hyperplasia, tumours, or hyperplasia plus tumours. Higher survival rates in the two higher groups may contribute to the higher number of ovarian tumours. The known modes of action via endocrine imbalance are unlikely, evidenced by the lack of interaction with the estrogen receptor and steroidogenesis by *in vitro* assays, no direct ovarian toxicity, and no reproductive abnormality. Furthermore, there was no accumulation or persistence of S-2200 and its metabolites in the ovary. Thus, the sex-cord stromal lesions are unlikely to be direct effects of treatment with S-2200 TG.

Based on these considerations, the increased incidence of the SCST observed in the 2-year rat study is not toxicologically significant. Therefore, the overall conclusion is that the data do not suggest a carcinogenic effect of mandestrobin and thus its classification is not warranted.

Comment of the RMS:

The RMS agrees with the conclusions drawn in this position paper.

Reference:	*In vitro* Steroidogenesis Assay of S-2200TG in H295R Cells
Author(s), year:	Kubo, H.; 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0065
Guideline(s):	OECD 456
GLP:	No
Deviations:	none
Validity:	Yes

MATERIAL AND METHODS:

Test Material:	S-2200 Technical Grade (S-2200 TG)
Lot/Batch:	ST-0811G (Batch specification 01)
Purity:	93.4%
Stability of test compound:	Expiry date: June 14th 2014 (after completion of treatment)

Control Materials:

Negative:	Solvent, DMSO
Positive:	Forskolin (10µM) for induction Prochloraz (1µM) for repression

Test organism: Human adrenocortical NCI-H295R cells (ATCC, Cat. No. CRL-2128)

Test concentrations: Based on the solubility and the potential interference with the hormone measurement systems, the maximum concentrations of S-2200TG for the initial run was set to 100 µM. In run 1, 100 µM of S-2200TG was cytotoxic (≤ 80% viability). Therefore the maximum concentration for subsequent runs was 30 µM.

(Run 1) 10 nM, 100 nM, 1 µM, 10 µM, 100 µM
(Run 2) 300 nM, 1 µM, 3 µM, 10 µM , 30 µM
(Run 3) 300 nM, 1 µM, 3 µM, 10 µM , 30 µM
(Run 4) 3 µM, 10 µM , 30 µM

Test procedure

In order to evaluate the effects of mandestrobin (S-2200 TG) on androgen and estrogen production, H295R cells cultured in 24-well plates were incubated with S-2200 TG in triplicate for 48 hours. For the evaluation of testosterone (T) and estradiol (E2) productions, four (Run 1, 2, 3 and 4) and three (Run 1, 2 and 3) independent experiments were performed, respectively. Dimethylsulfoxide (DMSO) was used as the vehicle at a final concentration of 0.1%.

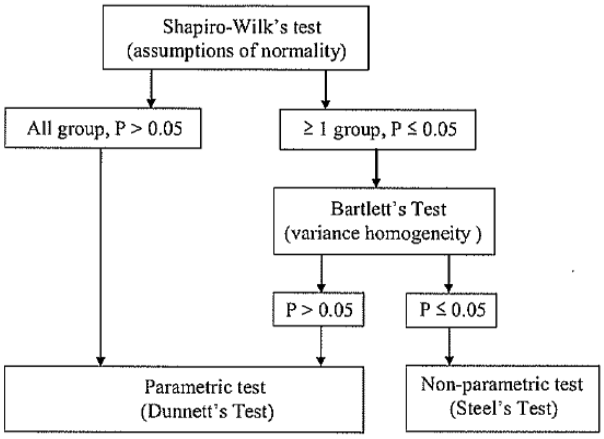
Positive controls (forskolin, a known inducer, and prochloraz, a known inhibitor) were evaluated concurrently with each run to confirm the changes in T and E2 levels in the assay.

T and E2 levels were measured using ELISA systems.

Cytotoxicity was evaluated after removal of the culture medium containing S-2200 TG using “Cell Counting Kit-8” (DOJINDO LABORATORIES).

Statistics:

To evaluate the relative increase or decrease in chemically altered hormone production, test chemical data were normalised to the mean solvent control values in the test plates. Furthermore, the results were normalised to cell viability when the cytotoxicity was less than 20%. The statistical analysis was carried according to the following procedure:



Differences were considered significant at $p \leq 0.05$.

Evaluation criteria:

According to the criteria laid down in the guideline, a chemical is judged to be positive if the induction is statistically different ($p \leq 0.05$) from the solvent control at two adjacent concentrations in at least two independent runs. A chemical is judged to be negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal (fold change at one concentration is statistically significantly different from the solvent control) or positive run. Results at concentrations exceeding the limits of solubility or at cytotoxic concentrations were not included in the interpretation of results.

FINDINGS:

10 µM of forskolin increased the levels of T and E2 more than 1.5-fold and 7.5-fold respectively, compared to the solvent control. 1 µM of prochloraz decreased both the levels of T and E2 less than 0.5-fold compared to the solvent control. These results indicated that present assay systems were validated by the positive control experiments, as required by the guideline.

In Run 1, S-2200 TG showed cytotoxicity to H295R cells at 100 µM. Therefore, the maximum S-2200 TG concentration was set to 30 µM in subsequent runs. No clear changes in T and E2 levels were observed in the assays (Run 1 through 4).

Table 70: Summary of cell viability (relative to solvent control), T production (relative to solvent control), and E2 production (relative to solvent control)

Concentration	Run 1			Run 2			Run 3			Run 4	
	T	E2	% Cell viability	T	E2	% Cell viability	T	E2	% Cell viability	T	% Cell viability
Solvent control	1.00	1.00	100.0	1.00	1.00	100.0	1.00	1.00	100.0	1.00	100.0
S-2200TG											

10 nM	1.03	1.17	106.2								
100 nM	1.02	1.11	107.2								
300 nM				1.00	1.03	109.1	1.02	1.07	97.4		
1 µM	1.00	1.10	102.6	1.01	1.14	107.1	1.01	1.09	94.5		
3 µM				0.92	1.21	99.0	0.96	1.06	96.7	1.04	95.5
10 µM	0.93	1.05	101.3	0.95	1.12	96.8	1.00	**1.21***	97.3	0.97	102.6
30 µM				**0.82***	1.09	98.2	1.01	1.03	82.8	0.98	81.1
100 µM			71.6								
Forskolin 10 µM	1.54	>15.07[a]	130.6	2.25	16.14	123.9	1.90	>12.50[a]	113.5	3.48	114.0
Prochloraz 1 µM	0.08	<0.24[a]	100.6	0.10	<0.26[a]	106.8	0.10	0.36	91.7	0.17	90.8

[a]: Measured OD value was out of the linear range of the standard curve. Fold-change was calculated from the Limit of quantification (31.25 and 500 pg/mL) normalised by cell viability.
* $P \leq 0.05$

CONCLUSIONS:

Testosterone production

Through Run 1 to 4, no significant differences in T production from the solvent control at two adjacent concentrations were detected. Judging from these results, it is concluded that S-2200 TG does not influence testosterone production in H295R cells at concentrations up to 30 µM.

Estradiol production

No significant differences in E2 production were observed in Run 1 or 2. Significant increases in E2 production was only found at 10 µM in Run 3, however significant increases were not detected at 3 or 30 µM. Judging from these results, all three Runs are considered negative. Therefore it is concluded that S 2200 does not influence E2 production in H295R cells at concentrations up to 30 µM.

The positive controls produced satisfactory results (i.e. induction 1.54-3.48 for T and > 12.50-16.14 for E2; inhibition: 0.08-0.17 for T and < 0.24-0.36 for E2), thus confirming the validity of the study.

Reference:	Evaluation of Effects of S-2200 TG and its Metabolites on Human Estrogen Receptor alpha and Human Androgen Receptor Using *in vitro*

	Reporter Gene Assays
Author(s), year:	Suzuki, N.; 2012
Report/Doc. number:	Sumitomo Chemical Co., Ltd. Report No. ROT-0066
Guideline(s):	OECD 455, Draft OECD Guideline 'Stably Transfected Human Androgen Receptor-α Transcriptional Activation Assay for Detection of Androgenic Agonist/ Antagonist Activity of Chemicals', Version 2010, Nov 25
GLP:	No
Deviations:	Both guidelines were combined and adapted to detect estrogen and androgen receptor agonistic and antagonistic activities of the test substances
Validity:	Yes

MATERIAL AND METHODS:

Test Materials:

S-2200 Technical Grade (S-2200 TG)
- Lot/Batch: ST-0811G (Batch specification 01)
- Purity: 93.4%

5-COOH-S-2200
- Lot/Batch: 252-001-55-2
- Purity: 99.7%

4-OH-S-2200
- Lot/Batch: CTS08026
- Purity: 99.9%

5-CH_2OH-S-2200
- Lot/Batch: CTS08030
- Purity: 99.9%

5-CA-S-2200-NHM
- Lot/Batch: CTS09005
- Purity: 94.9%

Control Materials:
- Negative: Solvent, DMSO
- Positive: 17β-Estradiol (E2) (purity > 97%) for hERα activation
  4-Hydroxytamoxifen (HTM) (purity > 98%) for hERα inhibition
  Dihydrotestosterone (DHT) (purity > 99%) for hAR activation
  Hydroxyflutamide (HFL) (purity > 98%) for hAR inhibition

Test organisms:
- For human estrogen receptor α (hERα) assays:
  hERα-HeLa-9903 (Stably transformed cell line derived from Human uterine cervix carcinoma HeLa cells)
- For human androgen receptor (hAR) assays:
  hAR-HeLa 4-11 (Stably transformed cell line derived from Human uterine cervix carcinoma HeLa cells)

Test concentrations:	Test chemicals were evaluated by cytotoxicity assays (100 pM -100 µM) Based on the results of the cytotoxicity assays, the maximum concentrations of the test substances in the receptor activation assays were:			
	S-2200 TG	hERα: 6 µM	hAR:	10 µM
	5-COOH-S-2200	hERα: 100 µM	hAR:	100 µM
	4-OH-S-2200	hERα: 10 µM	hAR:	10 µM
	5-CH_2OH-S-2200	hERα: 10 µM	hAR:	100 µM
	5-CA-S-2200-NHM	hERα: 100 µM	hAR:	100 µM

Cytotoxicity assays:

To determine the appropriate concentration ranges of test substances, cytotoxicity assays were carried out using hERα-HeLa-9903 and hAR-HeLa 4-11 cells. For detection of cell viability, 96 well microplates were prepared by adding test substances dissolved in DMSO at various concentrations.

hERα-HeLa-9903 or hAR-HeLa 4-11 cells were homogeneously suspended in DMEM (phenol free) containing 10% charcoal- treated FBS and plated to each well (n = 6).

After cell culturing at 37°C under 5% CO_2, the viability of the cells was measured by the CellTiter Glo Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. If cell viability was reduced by more than 20%, the concentrations were regarded as cytotoxic. The data at or above cytotoxic concentrations were excluded from the evaluation according to OECD TG 455.

Reporter gene assays to evaluate agonist and antagonist activities:

In agonist assays using hERα and hAR, positive controls (1 nM of E2 and DHT, respectively) and a vehicle control (DMSO) were analysed in each study.

hERα-HeLa-9903 or hAR-HeLa 4-11 cells were homogeneously suspended in DMEM (phenol free) containing 10% charcoal- treated FBS and plated to each well of 96 well plates (n = 6), and were exposed to serially diluted test substances in DMSO.

In antagonist assays using hERα and hAR, positive controls (HTM and HFL, respectively) and a vehicle control were analysed in each study. hERα-HeLa-9903 or hAR-HeLa 4-11 cells were plated in a manner similar to the agonist assays, and were exposed to serially diluted test chemicals in DMSO with typical hormones (100 pM of E2 and 100 pM of DHT, respectively).

After cell culturing for about 24 (for hERα) or 48 hours (for hAR) at 37°C under 5% CO_2, the medium was removed, and 50 µl of Steady-Glo Luciferase Assay System (Promega) was added to each well.

Cells were lysed by shaking for 30 minutes at room temperature in the dark, and the luciferase activity was measured using 96 wells format luminometer (TopCount NXT, Hewlett-Packard Company, Palo Alto, CA, USA). The luminescence data was converted into Excel format.

Data acceptance criteria:

In agonist assays, a study is judged to be acceptable when more than 4-fold or 10-fold of relative activity was observed at a concentration of 1 nM of E2 (for hERα) or DHT (for hAR), against the vehicle control according to the OECD TG 455 (for hERα) and/or our historical data (for hAR).

In antagonist assays, a study is judged to be acceptable when the value of more than 50% inhibition was observed at a concentration of 1 µM of HTM (for hERα) or HFL (for hAR), against the vehicle control according to our historical data.

Evaluation criteria:

In agonist assays, data analysis was performed for evaluation of test substances according to the OECD TG455 as follows:

a : mean values of a vehicle control (DMSO)

b : mean values of positive controls (1 nM of E2 or DHT)

c : mean values of test substances

B (relative activity of positive controls): b/a

C (relative activity of test substances): c/a

PC10: (B-1)/10+1

Judgment:

Positive: PC10 ≤ C Negative: PC10 > C

In antagonist assays, data analysis was performed according to a draft report of OECD guideline for the stably transfected human androgen receptor transcriptional assay for detection of androgenic agonist/antagonist activity of chemicals (Version 2010 Nov.25) as follows:

a : mean values of a vehicle control (DMSO)

b : mean values of positive controls (HTM or HFL)

c : mean values of test substances

C (relative activity of test substances): c/a ×100

Percent inhibition (positive controls): 100-B

Percent inhibition (test substances: 100-C

Judgment:

Positive: Percent inhibition ≥ 30 Negative: Percent inhibition < 30

FINDINGS:

Cytotoxicity assays:

The results are summarized in the following tables. As S-2200 TG was cytotoxic at 1 µM, a further assay was performed with concentrations between 1 and 10 µM S-2200 TG.

Table 71: hERα-HeLa-9903 viability (mean % relative to vehicle control, n = 6), first assay

Concentration	S-2200 TG	5-COOH-S-2200	4-OH-S-2200	5-CH_2OH-S-2200	5-CA-S-2200-NHM
0	100	100	100	100	100
100 pM	102	108	108	113	112
1 nM	110	112	115	119	116
10 nM	110	111	114	118	117
100 nM	109	112	114	118	118
1 µM	104	112	115	119	116
10 µM	**59***	110	94	114	115
100 µM	**29***	98	**36***	**72***	92

*Concentration excluded from analysis as cell viability was reduced ≥ 20% compared to solvent controls

Table 72: hERα-HeLa-9903 viability (mean % relative to vehicle control, n=6), second assay

Concentration	S-2200 TG
0	100
1 µM	107
2 µM	104
4 µM	95
6 µM	81
8 µM	**78***
10 µM	**58***

*Concentration excluded from analysis as cell viability was reduced ≥ 20% compared to solvent controls

Table 73: hAR-HeLa 4-11 viability (mean % relative to vehicle control, n=6), second assay

Concentration	S-2200 TG	5-COOH-S-2200	4-OH-S-2200	5-CH_2OH-S-2200	5-CA-S-2200-NHM
0	100	100	100	100	100
100 pM	103	102	101	103	102
1 nM	105	103	104	103	104

10 nM	104	102	102	104	102
100 nM	105	102	103	104	102
1 µM	104	102	103	104	103
10 µM	97	102	100	104	102
100 µM	**34***	99	**70***	96	98

*Concentration excluded from analysis as cell viability was reduced ≥ 20% compared to solvent controls

Agonist and Antagonist Assays for hERα:

The relative activity of a typical agonist E2 (1 nM) was more than 4-fold in both assays. This result satisfied the acceptance criteria.

The relative activities of S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and

5-CA-NHM-S-2200 did not exceed the PC10 values.

It was thus concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and

5-CA-S-2200-NHM had no agonistic activities towards hERα.

Table 74: Agonistic activities (mean ratio of activity to vehicle control, n = 6) of S-2200 TG and its metabolites to hERα, first assay

Concentration	**Positive control E2**	**S-2200TG**	**5-COOH-S-2200**	**4-OH-S-2200**	**5-CH_2OH-S-2200**	**5-CA-S-2200-NHM**
0	1.00	1.00	1.00	1.00	1.00	1.00
10 pM	**2.20***	NT	NT	NT	NT	NT
100 pM	**4.16***	0.99	1.05	1.07	0.93	1.11
1 nM	**6.30***	1.04	1.00	0.97	0.93	0.98
10 nM	**5.96***	1.13	1.04	0.96	0.97	0.94
100 nM	**4.99***	1.02	0.95	1.00	0.98	1.17
1 µM	**4.78***	1.01	1.09	1.09	0.90	1.09
10 µM	NT	NT	1.01	1.14	0.87	1.27
100 µM	NT	NT	1.13	NT	NT	0.94

*Positive effect was observed (criterion: relative activity exceeds the PC10 value of 1.53)
NT = Not tested. E2 = 17β-estradiol

Table 75: Agonistic activities (mean ratio of activity to vehicle control, n = 6) of S-2200 TG and its metabolites to hERα, second assay

Concentration of S-2200 TG	**mean ratio of activity to control**	**Concentration of positive control E2**	**mean ratio of activity to control**

0 µM	1.00	**0**	1.00
1 µM	1.08	**10 pM**	**1.82***
2 µM	1.11	**100 pM**	**4.13***
4 µM	1.17	**1 nM**	**5.46***
6 µM	1.18	**10 nM**	**5.22***
8 µM	NT	**100 nM**	**5.63***
10 µM	NT	**1 µM**	**4.77***
		10 µM	NT

*Positive effect was observed (criterion: relative activity exceeds the PC10 value of 1.45)
NT = Not tested. E2 = 17β-estradiol

For measurement of antagonistic activities, the cells were exposed to the various chemicals and the typical activator E2 at 100 pM.

Since more than 50% inhibition value was observed at a concentration of 1 µM of HTM, the assay system was validated.

The inhibition caused by S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-NHM did not exceed 30% of the inhibition caused by 1 µM HTM in both assays.

Therefore, it was concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-S-2200-NHM had no antagonistic activities towards hERα.

Table 76: Antagonistic activities (mean % inhibition relative to vehicle control, n = 6) of S-2200 TG and its metabolites to hERα, first assay

Concentration	**Positive control HTM**	**S-2200TG**	**5-COOH-S-2200**	**4-OH-S-2200**	**5-CH_2OH-S-2200**	**5-CA-S-2200-NHM**
0	0	0	0	0	0	0
10 pM	2	NT	NT	NT	NT	NT
100 pM	-34	-2	-16	-17	-22	-17
1 nM	-28	-17	-10	-14	-10	-41
10 nM	**42***	-11	-11	-2	3	-50
100 nM	**52***	-8	-6	-20	-20	-43
1 µM	**59***	-19	-9	4	-19	-47
10 µM	**72***	NT	-17	0	1	-28
100 µM	NT	NT	14	NT	NT	-5

*Positive effect was observed (criterion: antagonistic activity is equal to or exceeds 30% of 1 µM HTM
NT = Not tested. HTM = 4 Hydroxytamoxifen

Table 77: Antagonistic activities (mean % inhibition relative to vehicle control, n = 6) of S-2200 TG and its metabolites to hERα, second assay

Concentration of S-2200TG	mean ratio of activity to control	Concentration of positive control HTM	mean ratio of activity to control
0 µM	0	0	0
1 µM	1	10 pM	-5
2 µM	-10	100 pM	-27
4 µM	1	1 nM	-33
6 µM	-8	10 nM	**48***
8 µM	NT	100 nM	**62***
10 µM	NT	1 µM	**62***
		10 µM	**72***

*Positive effect was observed (criterion: antagonistic activity is equal to or exceeds 30% of 1 µM HTM
NT = Not tested. HTM = 4 Hydroxytamoxifen

Agonist and Antagonist Assays for hAR:

More than 10-fold induction of relative activity was observed at 1 nM of DHT, thus the acceptance criteria were satisfied.

The relative activities of S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CAS-2200-NHM did not exceed PC10 values.

It was, therefore, concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-S-2200-NHM had no agonistic activities to hAR.

Table 78: Agonistic activities (mean ratio of activity to vehicle control, n=6) of S-2200 TG and its metabolites to hAR

Concentration	Positive control DHT	S-2200 TG	5-COOH-S-2200	4-OH-S-2200	5-CH_2OH-S-2200	5-CA-S-2200-NHM
0	1.00	1.00	1.00	1.00	1.00	1.00
10 pM	1.86	NT	NT	NT	NT	NT
100 pM	**13.67***	0.95	0.96	0.97	0.95	0.98
1 nM	**24.89***	0.94	0.96	0.95	0.94	0.95
10 nM	**26.10***	0.91	0.94	0.93	0.93	0.91
100 nM	**25.82***	0.89	0.94	0.90	0.90	0.90
1 µM	**27.25***	0.93	0.91	0.93	0.92	0.93
10 µM	**24.15***	1.01	0.90	1.00	0.92	0.93

100 µM	NT	NT	0.99	NT	0.81	1.01

* - Positive effect was observed (criterion: relative activity exceeds the PC10 value of 3.39)
NT = Not tested. DHT = Dihydrotestosterone

For measurement of antagonistic activities, the cells were exposed to the various chemicals and the typical activator DHT at 100 pM.

Since more than 50% inhibition of activation was observed at 1 µM of HFL, the assay system was validated.

The percent inhibition values of S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-S-2200-NHM did not exceed 30% of the inhibition caused by 1 µM HFL.

It was, therefore, concluded that S-2200 TG, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-S-2200-NHM had no antagonistic activities to hAR.

Table 79: Antagonistic activities (mean % inhibition relative to vehicle control, n=6) of S-2200 TG and its metabolites to hAR

Concentration	**Positive control HFL**	**S-2200 TG**	**5-COOH-S-2200**	**4-OH-S-2200**	**5-CH_2OH-S-2200**	**5-CA-S-2200-NHM**
0	0	0	0	0	0	0
10 pM	6	NT	NT	NT	NT	NT
100 pM	12	8	7	8	8	7
1 nM	14	9	8	13	12	12
10 nM	**31***	13	11	11	15	13
100 nM	**69***	12	10	13	16	12
1 µM	**88***	12	11	12	15	13
10 µM	**79***	16	8	12	9	11
100 µM	NT	NT	13	NT	15	11

CONCLUSIONS:

Mandestrobin (S-2200 TG) and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-S-2200-NHM) did not show agonistic or antagonistic effects on hERα and hAR – induced transcriptional activation under the conditions of the present study.

4.12.1.4 Human information

A formal statement from the manufacturer is presented (Nishioka, K.; 2012), that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff. No poisoning incidents or clinical cases have been reported. Mandestrobin is a new

chemical currently in development. There has been no exposure of the general population or epidemiology study.

4.12.2 Summary and discussion

Summary of neurotoxicity:

In a range-finding assay designed to identify the time of peak effect after an acute oral limit dose (2000 mg/kg bw) of mandestrobin, no reaction was seen, so no peak effect was evident. In the subsequent definitive acute neurotoxicity study, investigations intended for the "time of peak effect" were therefore conducted at the latest timepoint compatible with guideline, i.e. 8 hours post-dose.

The only treatment-related finding in the definitive acute neurotoxicity study was a decrease in mean locomotor activity, seen at the top dose (2000 mg/kg bw) at 8 hours post-dose. In the absence of any other specific neurological findings, this is probably attributable to transient systemic toxicity rather than typical neurotoxicity. The NOAEL, set on a highly precautionary basis assuming a decrease in locomotor activity in absence of any other neurological change might be meaningful, was 1000 mg/kg bw.

In a 90-day subchronic neurotoxicity study, at doses of up to 15000 ppm, no evidence of specific neurotoxicity was found. The NOAEL for specific neurotoxicity was therefore approximately 1024 mg/kg bw/day, i.e. the highest dose level tested. On the basis of body weight gain retardation at the top dose in males only, the NOAEL for systemic toxicity was 338 mg/kg bw/day.

Summary of immunotoxicity:

In an oral range finding study and in an immunotoxicity study (both 28-day, rats), no treatment related effects were observed up to the highest dose tested. The NOAEL for immunotoxicity was therefore set at 15000 ppm (equivalent to 1419 mg/kg bw/day).

Summary of mechanistic studies:

Several *in vivo* and *in vitro* mechanistic studies were performed, and two detailed position papers were submitted dealing with (i) liver and thyroid effects and (ii) ovary issues.

Two *in vivo* studies were performed (one in rats, one in mice) to gain insight on the mechanistic basis of the liver and thyroid effects observed in the main studies in rats. The effects observed were compared to CAR-mediated induction of liver enzymes and subsequent perturbations of thyroid hormones, a well-studied mode of action with no relevance for human risk assessment.

A detailed description of the effects observed in the main and mechanistic studies, as well as a detailed assessment of the mode of action of mandestrobin is provided in a position paper included in this report.

The proposed phenobarbital-like mode of action for mandestrobin tested in the rat mechanistic *in vivo* study is considered to satisfy the Bradford Hill criteria of dose and temporal concordance, biological plausibility, coherence, strength, consistency, and specificity for thyroid follicular cell hypertrophy. Although the phenobarbital–like mode of action could theoretically operate in humans, the markedly different susceptibility for thyroid abnormality render it non relevant for

humans. Furthermore, no increased tumour rates were observed up to the highest doses tested in the long term/carcinogenicity studies.

Two *in vitro* studies were performed to address possible hormonal effects of mandestrobin and its metabolites. Mandestrobin had no influence on testosterone and estradiol production, and mandestrobin and its metabolites 5-COOH-S-2200, 4-OH-S-2200, 5-CH_2OH-S-2200 and 5-CA-S-2200-NHM did not have any effects on estrogen or androgen mediated reporter gene activity.

In a position paper (and its update), the toxicological significance of the slightly higher incidence of benign ovarian sex-cord stromal tumours in the 2-year rat study is discussed based on existing data of mandestrobin, background data of ovarian proliferative change present in elderly rats, and published information. The overall conclusion is that the data do not suggest a carcinogenic effect of mandestrobin and thus classification is not warranted.

4.12.3 Comparison with criteria

According to the available studies, there was no indication of a neurotoxic or immunotoxic potential of mandestrobin.

Mechanistic data indicates that mandestrobin is a hepatic enzyme inducer via at least constitutive androstane receptor (CAR) activation in rat, in a manner similar to phenobarbital (PB). However, during the pesticide peer review data were considered insufficient, as phenobarbital induced cetrilubular hypertrophy, whereas the hepatocyte hypertrophy induced by mandestrobin was diffuse.
In repeat dose toxicity studies the primary target organ of mandestrobin is the liver in all species examined. The thyroid was also a target organ in the rat, but not in the mouse and dog. The primary liver finding in the rat was hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the main thyroid finding was follicular cell hypertrophy. However, no tumourigenicity was observed in rat or mouse carcinogenicity studies that would trigger classification.

Mandestrobin had no influence on testosterone and estradiol production, and mandestrobin and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH_2OH-S-2200 and 5-CA-S-2200-NHM) did not have any effects on estrogen or androgen mediated reporter gene activity.

In a position paper (and its update), the toxicological significance of the slightly higher incidence of benign ovarian sex-cord stromal tumours in the 2-year rat study is discussed based on existing data of mandestrobin, background data of ovarian proliferative change present in elderly rats, and published information. The overall conclusion is that the data do not suggest a carcinogenic effect of mandestrobin and thus classification is not warranted.

4.12.4 Conclusions on classification and labelling

No classification and labelling is proposed for mandestrobin regarding neurotoxicity, immunotoxicity or other effects in repeated dose toxicity studies.

5 ENVIRONMENTAL HAZARD ASSESSMENT

The active substance S-2200 (mandestrobin) is a racemic mixture (1:1) of the two isomers S-2167 (S-2200 *R*-isomer) and S-2354 (S-2200 *S*-isomer). In order to determine the route and rate of degradation of the active substance mandestrobin (S-2200) in soil and water separate studies with the two isomers were conducted. No isomerisation between the S-2200 *R*- and *S*-isomers was observed in any of the studies.

5.1 Degradation

Table 80: Summary of relevant information on degradation

Method	Results	Remarks	Reference
Hydrolysis Guideline: OECD 111, EEC Method C.7 , OPPTS 835-2110, J MAFF Nousan 8147, section 2-6-1 (2001)	Mandestrobin, [Benzyl-^{14}C]S-2167 (S-2200 *R*-isomer),: DT50 (pH 4, 50 °C): stable to hydrolysis DT50 (pH 7, 50 °C): stable to hydrolysis DT50 (pH 9, 50 °C): stable to hydrolysis	radiochemical purity: > 98 % (HPLC), chiral purity: 100 %,	Lewis, C.J., Aldermann, D., 2010a Report No.: 8200206; ROM-0005
Hydrolysis Guideline: OECD 111, EEC Method C.7 , OPPTS 835-2110, J MAFF Nousan 8147, section 2-6-1 (2001)	Mandestrobin, [Benzyl-^{14}C]S-2354 (S-2200 *S*-isomer), DT50 (pH 4, 50 °C): stable to hydrolysis DT50 (pH 7, 50 °C): stable to hydrolysis DT50 (pH 9, 50 °C): stable to hydrolysis	radiochemical purity: > 98 % (HPLC)	Lewis, C.J., Aldermann, D., 2010b Report No.: 8200194; ROM-0006
Photolysis Guideline: OECD 316 (October 2008), 95/36/EC, 94/37/EC, US-EPA N 161-2 (1982), J MAFF Nousan-8147 section 2-6-2 (2000)	Mandestrobin, [Benzyl-^{14}C]S-2167 and [Phenoxy-^{14}C]S-2167 (S-2200 *R*-isomer),: DT_{50} values (also equivalent to natural summer sunlight in UK/US) of 4.4 days S-2200-OR (maximum 24.0 % and 21.6 % AR at DAT 7) and S-2200-ORC (maximum 16.0 % and 17.7 % AR at DAT 30 and 21)	radiochemical purity: ≥ 98 % (HPLC)	Lewis, C.J., Aldermann, D., 2010 c Report No: 8200199; ROM-0013

Method	Results	Remarks	Reference
Photolysis Guideline: OECD 316 (2008), 95/36/EC, 94/37/EC, US-EPA N 161-2 (1982), J MAFF Nousan-8147 section 2-6-2 (2000)	Mandestrobin, [Benzyl-^{14}C]S-2354 (S-2200 *S*-isomer),: DT_{50} values (also equivalent to natural summer sunlight in UK/US) of 4.6 days S-2200-OR (maximum 18.6 % at DAT 7) and S-2200-ORC (maximum 10.5 % at DAT 14 and at DAT 30)	radiochemical purity: ≥ 98 % (HPLC)	Lewis, C.J., Aldermann, D., 2010 d Report No: 8200195; ROM-0011
Biological degradation Guideline: OECD 301 B Ready Biodegradability (Adopted 1981, Revised 1992)	Not ready biodegradable	purity 93.4 %	Graham, R., 2009 Report No. 1002463; ROM-0003
Water/Sediment Study Guideline: OECD Guideline 308 (2002)	Mandestrobin, [Benzyl-^{14}C]S-2167 and [Phenoxy-^{14}C]S-2167 (S-2200 *R*-isomer): Water (2 systems): DT50: 9/7/15/17 d DT90: 83/56/333/416 d Whole system (2 systems): DT50: 342/344/284/654 d DT90: 942 - >1000 d	radiochemical purity: > 99 %	Graham, R., 2011a Report No: 8200193; ROM-0022
Water/Sediment Study Guideline: OECD Guideline 308 (2002)	Mandestrobin, [Benzyl-^{14}C]S-2354 (S-2200 *S*-isomer) Water (2 systems): DT50: 7/23 d DT90: 60/169 d Whole system (2 systems): DT50: 155/600 d DT90: 516/>1000 d	radiochemical purity: > 99 %	Graham, R., 2011b Report No: 8200200, ROM-0023
Kinetic Evaluation of the Aerobic Aquatic metabolism - Calculation of S-2200 sediment water kinetics	The data of the *S*-isomer and of the *R*-isomer were combined to determine the rate of degradation of S-2200 (the racemate): Water (2 systems): DT50: 7.8 / 19 d DT90: 64.8 / 157.7 d Whole system (2 systems): DT50: 212 / 519.1 d (332 d: geometric mean) DT90: 703 / 1725 d		Jarvis, T., Mamouni, A., 2012 Report No: ROM-0034

5.1.1 Stability

Hydrolysis:

Studies on the hydrolytic degradation were conducted with S-2167 and S-2354 at pH 4, 7 and 9, [benzyl-^{14}C] label.

Reference:	**[^{14}C]S-2167 (S-2200 *R*-isomer): Hydrolytic Stability**
Author(s), year:	Lewis, C.J., Aldermann, D., 2010a
Study/report number:	8200206; ROM-0005
Guideline(s):	OECD 111 (April 2004), EC Directive 94/37/EC, Section 2.9.1 (July 1994) EEC Method C.7 - Abiotic degradation. Hydrolysis as a function of pH (1992), OPPTS 835-2110 – Hydrolysis as a function of pH (1998), Japan MAFF New Test Guideline 12-Nousan 8147, section 2-6-1 and 2-9-13 (2000)
GLP:	Yes
Deviations:	None
Validity:	Study considered acceptable

Material and methods:

Test substance:	• [Benzyl-^{14}C]S-2167 (S-2200 *R*-isomer), 4.22 GBq mmol^{-1}, > 98 % radiochemical purity (HPLC), chiral purity: 100 %, batch RIS2008-010 • Unlabelled S-2167, 100 % chemical purity
Reference substances:	S-2354 (unlabelled), 2-COOH-S-2200, 5-COOH-S-2200, S-2200-OR, S-2200-ORC, De-Xy-S-2200, DX-CA-S-2200, chemical purity: >99 %; S-2200-PR, chemical purity: 89.6 % MCBX, chemical purity: 96.9 %
Test systems:	• pH 4: 0.05 M phthalate buffer (potassium phthalate solution adjusted with sodium hydroxide or hydrochloric acid) • pH 7: 0.05 M phosphate buffer (potassium dihydrogen phosphate solution adjusted with sodium hydroxide or hydrochloric acid) • pH 9: 0.05 M borate buffer (sodium tetraborate solution adjusted with sodium hydroxide or hydrochloric acid) All buffers sterilized by autoclaving. Oxygen content reduced by sonication and nitrogen bubbling.
Volatile traps:	No volatile traps (no volatiles expected – confirmed by complete material balance).
Test temperature:	50 °C (pH 4 and pH 7 and pH 9)
Test duration:	Up to 5 days in the dark.
Sample concentration:	1 mg L^{-1}
Co-solvent:	Acetonitrile.
Analysis:	LSC, HPLC-UV, TLC (selected samples), chiral HPLC LOD ca. 0.3 % of AR (HPLC)
Kinetic evaluation:	Not applicable, no degradation

Findings:

Mean material balances of all experiments were in a range of 102.0 – 105.0 % of AR. Owing to the complete mass balance $^{14}CO_2$ formation is considered to be negligible. The product balance is presented in table 81.

No degradation of S-2167 (S-2200 *R*-isomer) was observed and no isomerisation of S-2167 (S-2200 *R*-isomer) to S-2354 (S-2200 *S*-isomer) could be observed.

Table 81: Product balance following hydrolysis of [14C] S-2167 in pH 4, 7 and 9 buffer at 50°C

pH	Time [days]	label	S-2167 S-2200 *R*-isomer	Unknowns	Unresolved background	Mass balance
4	0	Benzyl	102.2	ND	0.9	103.1
	0.1	Benzyl	103.1	ND	0.1	103.3
	5	Benzyl	104.5	ND	0.4	105.0
7	0	Benzyl	101.7	ND	0.5	102.2
	0.1	Benzyl	101.5	ND	0.7	102.2
	5	Benzyl	103.1	ND	0.4	103.5
9	0	Benzyl	101.9	ND	0.2	102.2
	0.1	Benzyl	101.7	ND	0.3	102.0
	5	Benzyl	102.9	ND	0.2	103.1

Conclusion:

S-2167 (S-2200 *R*-isomer) was hydrolytically stable at 50°C at pH 4 and 7 and 9 over 5 days. No isomerisation of S-2167 (S-2200 *R*-isomer) to S-2354 (S-2200 *S*-isomer) could be observed.

Reviewer comments:

None.

Reference:	**[^{14}C]S-2354 (S-2200 *S*-isomer): Hydrolytic Stability**
Author(s), year:	Lewis, C.J., Aldermann, D., 2010b
Study/report number:	8200194; ROM-0006
Guideline(s):	OECD 111 (April 2004), EC Directive 94/37/EC, Section 2.9.1 (July 1994) EEC Method C.7 - Abiotic degradation. Hydrolysis as a function of pH (1992), OPPTS 835-2110 – Hydrolysis as a function of pH (1998), Japan MAFF New Test Guideline 12-Nousan 8147, section 2-6-1 and 2-9-13 (2000)
GLP:	Yes
Deviations:	None
Validity:	Study considered acceptable

Material and methods:

Test substance:
- [Benzyl-^{14}C]S-2354 (S-2200 *S*-isomer), 4.22 GBq mmol^{-1}, > 98 % radiochemical purity (HPLC), batch RIS2008-009
- S-2354 (unlabelled), chemical purity: >99 %

Reference substances: S-2167 (unlabelled), 2-COOH-S-2200, 5-COOH-S-2200, S-2200-OR, S-2200-ORC, De-Xy-S-2200, DX-CA-S-2200, chemical purity: >99 %; S-2200-PR, chemical purity: 89.6 % MCBX, chemical purity: 96.9 %

Test systems:
- pH 4: 0.05 M phthalate buffer (potassium phthalate solution adjusted with sodium hydroxide or hydrochloric acid)

- pH 7: 0.05 M phosphate buffer (potassium dihydrogen phosphate solution adjusted with sodium hydroxide or hydrochloric acid)
- pH 9: 0.05 M borate buffer (sodium tetraborate solution adjusted with sodium hydroxide or hydrochloric acid)

All buffers sterilized by autoclaving. Oxygen content reduced by sonication and nitrogen bubbling.

Volatile traps: No volatile traps (no volatiles expected – confirmed by complete material balance).

Test temperature: 50 °C (pH 4 and pH 7 and pH 9)

Test duration: Up to 5 days in the dark.

Sample concentration: 1 mg L^{-1}

Co-solvent: Acetonitrile.

Analysis: LSC, HPLC-UV, chiral HPLC, 2D-TLC (selected samples)
LOD ca. 0.2 % of AR (HPLC)

Kinetic evaluation: Not applicable, no degradation

Findings:

Mean material balances of all experiments were in a range of 98.7-100.4% of AR. Owing to the complete mass balance $^{14}CO_2$ formation is considered to be negligible. The product balance is presented in table 82.

No degradation of S-2354 (S-2200 *S*-isomer) was observed and no isomerisation of S-2354 (S-2200 *S*-isomer) to S-2167 (S-2200 *R*-isomer) could be observed.

Table 82: Product balance following hydrolysis of [14C]S-2354 in pH 4, 7 and 9 buffer at 50°C

pH	Time [days]	label	S-2354 S-2200 *R*-isomer	Unknowns	Unresolved background	Mass balance
4	0	Benzyl	99.8	ND	0.5	100.4
	0.1	Benzyl	98.9	ND	0.6	99.5
	5	Benzyl	98.9	ND	0.5	99.4
7	0	Benzyl	98.7	ND	0.7	99.4
	0.1	Benzyl	98.2	ND	0.6	98.8
	5	Benzyl	99.0	ND	0.5	99.5
9	0	Benzyl	99.2	ND	0.5	99.7
	0.1	Benzyl	98.0	ND	0.7	98.7
	5	Benzyl	99.1	ND	0.3	99.4

Conclusion:

S-2354 (S-2200 *S*-isomer) was hydrolytically stable at 50°C at pH 4 and 7 and 9 over 5 days. No isomerisation of S-2354 (S-2200 *S*-isomer) to S-2167 (S-2200 *R*-isomer) could be observed.

Reviewer comments:

None

Photolysis:

Studies on the photolytic degradation of S-2200 were conducted with S-2167 (S-2200 *R*-isomer, [benzyl-^{14}C] and [phenoxy-^{14}C] labelled) and S-2354 (S-2200 *S*-isomer, [benzyl-^{14}C] labelled) in sterile buffered water (pH 7.0).

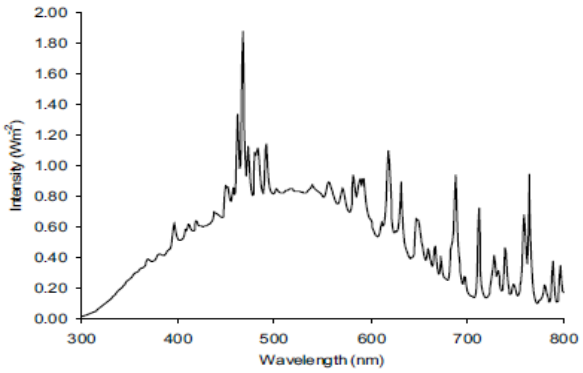
Quantum yield and half-life time under environmental conditions were determined for S-2167 and S-2354.

Reference:	**[^{14}C]S-2167 (S-2200 *R*-isomer): Photodegradation and Quantum Yield in Sterile, Aqueous Solution.**
Author(s), year:	Lewis, C.J., Aldermann, D., 2010 c
Study/report number:	8200199; ROM-0013
Guideline(s):	OECD 316 (October 2008), 95/36/EC, 94/37/EC, US-EPA N 161-2 (1982), J MAFF Nousan-8147 section 2-6-2 (2000)
GLP:	Yes
Deviations:	None
Validity:	Study considered acceptable

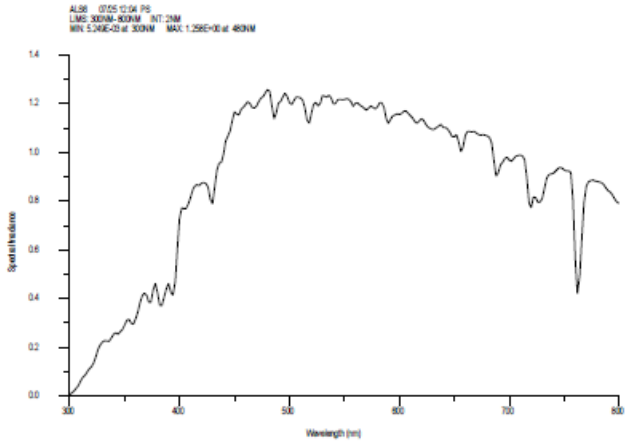
Material and methods:

Test substances:	• [Benzyl-^{14}C]S-2167, 4.22 GBq mmol^{-1} (13.5 MBq mg^{-1},) ≥ 98 % radiochemical purity (HPLC), batch RIS2008-010, 100 % optical purity • [Phenoxy-^{14}C]S-2167, 4.44 GBq mmol^{-1} (14.2 MBq mg^{-1}) ≥ 98 % radiochemical purity (HPLC), batch RIS2009-002, 100 % optical purity
Reference substances:	S-2167 (unlabelled), S-2354 (unlabelled, S-2200 S-isomer), 5-COOH-S-2200, 2-COOH-S-2200, S-2200-ORC, S-2200-OR, De-Xy-S-2200, DX-CA-S-2200, 2,5-dimethylphenol, (all: purity > 99 %); S-2200-PR (purity: 89.6 %), MCBX (purity: 96.9 %),
Test system:	Sterile pH 7.0 buffer (0.01 M phosphate buffer, autoclaved), quartz glass units, sterility was checked throughout the experiment. A PNAP/PYR binary actinometer for determination of quantum yield.
Test temperature:	25 ± 1 °C
Test duration:	30 days continuous irradiation (30 days incubation equivalent to ca. 30 summer sunlight days in US and UK) or dark incubation.
Sample concentration:	1.0 µg mL^{-1}
Co-solvent:	Acetonitrile
Test system:	Xenon lamp (Suntest Accelerated Exposure machine), cut-off < 290 nm, ca. 25 watts m^{-2} (300 – 400 nm).

Radiation Spectrum (300 to 800 nm) of the Xenon Lamp in Suntest Accelerated Exposure Machine 6 (position 5)



Radiation spectrum (300 to 800 nm) of Harrogate (*ca* 54°N) summer sunlight on 25 July 1995



Spectrum of experimental radiation is close to spectral distribution of natural sunlight (solar irradiation of Harrogate in summer).

Volatile traps:	Polyurethane foam bung, 1 x ethanediol trap and 2 x 2 M NaOH trap
Analysis:	LSC, HPLC-UV, 2D-TLC LOD < 1 % of AR (HPLC)
Kinetic evaluation:	Simple first order (SFO) kinetics, Microsoft Excel, KinGUI 1.1,

Findings:

Sterile conditions were maintained throughout the study. Mass balance was in a range of 91 to 102 % of AR for all experiments. Unit rinses contained ≤ 1.7 % of AR for irradiated samples and ≤ 3.5 % AR for dark samples and hence confirmed no adsorption of radioactivity to the glass vessels. The polyurethane foam bungs and ethanediol traps from the incubated samples contained ≤1.2 % of AR during the entire incubation period. The NaOH traps contained up to 1.3 % of AR from the [benzyl-^{14}C]S-2167 solution after 30 days and up to 8.1 % of AR from the [penoxy-^{14}C] labelled S-2167 solution after 30 days (phenoxy labelled solution: confirmed as CO_2 by precipitation after addition of barium chloride solution). Under irradiation S-2167 was subjected to extensive photolytic rearrangement procedures, resulting in two major metabolites: S-2200-OR (maximum 24.0 % of AR at DAT 7 for [benzyl-^{14}C] label and 21.6 % of AR at DAT 7 for [phenoxy-^{14}C] label) and S-2200-ORC (maximum 16.0 % of AR at DAT 30 for [benzyl-^{14}C] label and 17.7 % of AR at DAT 21 for [phenoxy-^{14}C] label). Other identified products were S-2200-PR (max. 9.6% after 4 days) and De-Xy-S-2200 (max. 4.6 % after 21 days). In addition,

trace amounts (≤1.5%) of 5-COOH-S-2200, MCBX and DX-CA-S-2200 were tentatively identified but could not be confirmed by TLC. A very large number of unknown peaks (up to 33) were found which individually reached up to 6.8 % AR (30 days) for benzyl and 6.6 % AR (14 days) for phenoxy label.

Without irradiation no degradation of S-2167 was observed. At all sampling intervalls ≥ 93 % of AR was recovered as S-2167 and no metabolites > 1 % AR were detected. No S-2354 was present in any sample (chiral HPLC) and therefore no isomerisation of S 2167 occurred. The results of HPLC analyses are shown in Table 83. The proposed route of degradation is shown in Figure 1.

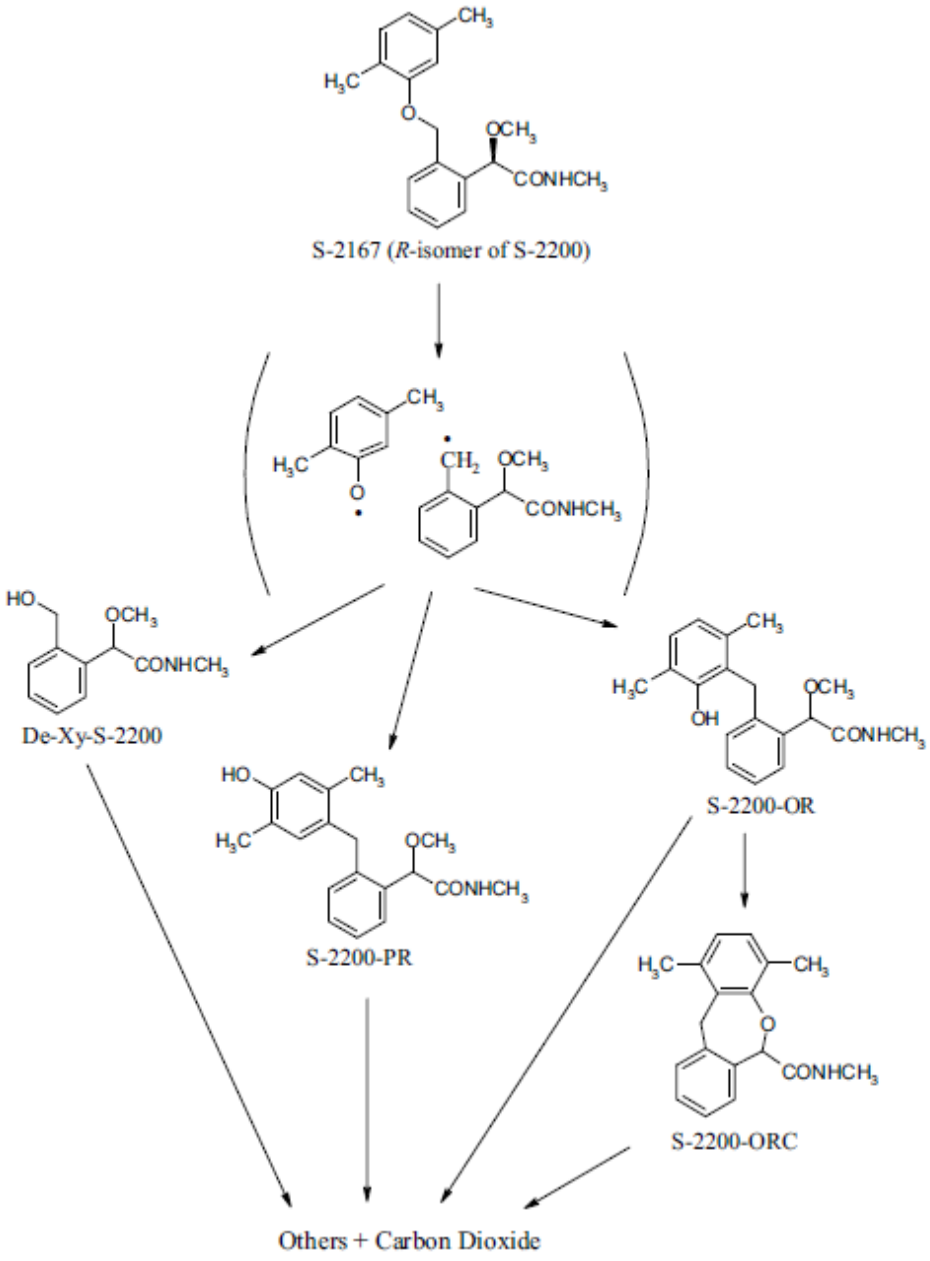


Figure 1: Proposed photolysis degradation route of S-2167 (S-2200 R-isomer)

Table 83: Photo-transformation of S-2167 in sterile water buffered at pH 7 [% of AR].

Label	Conditions	DAT	S-2167	S-2200-OR	S-2200-ORC	S-2200-PR	De-Xy-S-2200	Peak A*	Total unknowns (number)	Largest unknown	Organic volatiles	CO_2	Mass balance
[Benzyl-^{14}C]	**Irradiated**	**0**	97.1	ND	ND	ND	ND	ND	ND	NA	NA	NA	99.0
		1	83.1	5.7	0.1	3.8	0.6	ND	2.2 (3)	1.6	0.2	ND	99.1
		2	72.6	11.3	0.7	6.4	0.5	0.6	4.2 (3-7)	2.3	0.6	ND	99.2
		4	61.2	18.7	1.9	9.3	0.5	1.2	5.8 (5)	3.2	ND	ND	101.6
		7	39.3	24.0	5.7	9.2	2.6	4.7	11.5 (9-10)	3.3	ND	ND	99.8
		14	11.8	17.4	12.3	3.2	2.8	7.3	39.3 (16-19)	5.2	0.3	0.4	98.4
		21	4.9	6.9	10.7	1.6	4.6	6.8	58.9 (25)	6.5	0.1	1.1	99.2
		30	2.5	6.7	16.0	1.6	4.5	7.2	56.3 (23-26)	6.8	0.2	1.3	99.9
	Dark	**30**	93.8	ND	ND	ND	ND	ND			0.7	ND	99.3
[Phenoxy-^{14}C]	**Irradiated**	**0**	98.7	ND	ND	ND		ND	ND	NA	NA	NA	100.6
		1	85.6	6.5	ND	4.1		1.2	1.6 (1)	1.8	0.2	ND	101.7
		2	69.9	13.1	0.9	7.5		2.9	3.9 (1-5)	2.9	0.6	0.1	101.6
		4	44.8	19.8	3.4	9.6		4.1	14.7 (9-19)	3.8	0.6	0.4	100.7
		7	26.2	21.6	8.2	8.5		5.3	25.4 (15-17)	3.7	0.6	1.0	100.7
		14	7.1	15.6	16.5	3.6		4.5	43.4 (25-29)	6.6	0.9	2.3	98.1
		21	1.7	4.7	17.7	1.5		5.0	53.3 (26-27)	4.9	0.8	6.7	95.0
		30	0.5	2.4	12.2	1.2		4.9	57.0 (28-33)	5.8	0.8	8.2	90.7
	Dark	**30**	96.0	ND	ND	ND		ND			0.1	0.1	98.7

* consists of several peak, each ≤ 2.5 % of AR (benzyl labelled) or ≤ 4.1 % AR (phenoxy labelled),

ND: not detected

Table 84: Calculated aquatic photolytic DT50 and DT90 [days] of S-2167 and metabolites using SFO kinetics at pH 7.

Compound	Label	DT_{50} (UK/US equivalent)	DT_{90} (UK/US equivalent)	Chi2-error
S-2167	Benzyl	5.3	17.5	3.69
	Phenoxy	3.6	12.0	2.64
	Both	4.4	14.6	1.16
S-2200-OR	Both	5.1	16.9	9.89
S-2200-PR	Both	2.5	8.3	9.23

The DT_{50} values determined for S-2167 are equivalent to US and UK summer sunlight. The first order DT_{50} value for S-2167 was 4.4 days. The DT_{50} values for the metabolites were determined by modelling. A mean SFO DT_{50} value of 5.1 days was calculated for S-2200-OR, whereas S-2200-ORC appeared stable to photolysis. The DT_{50} of S-2200-PR was ca. 2.5 days.

The quantum yield of S-2167 was determined by direct comparison of the degradation rates of S-2167 and of PNAP in a binary actinometer, both exposed to identical lighting conditions. The quantum yield value for S-2167 was determined to be 0.283.

Conclusion:

Under irradiated experimental conditions, S-2167 degraded at a first order DT_{50} value which corresponds to about 4.4 days under environmental conditions in US/UK summer (reference Harrogate 54 °N). Appropriate controls confirmed that there was no degradation in darkness. No isomerisation from S-2200 *R*-isomer (S-2167) to S-2200 *S*-isomer (S-2354) occurred. The main photolytic metabolites were S-2200-OR (max. 24 % after 7 days) and S-2200-ORC (max 17.7 % after 21 days). A mean SFO DT_{50} value of 5.1 days was calculated for S-2200-OR, whereas S-2200-ORC appeared stable to photolysis. Other products identified were S-2200-PR (max. 9.6 % AR after 4 days) and De-Xy-S-2200 (max. 4.6 % AR after 21 days). The DT_{50} of S-2200-PR was calculated to be 2.5 days. Volatile radioactivity comprised < 10 % AR. Extensive breakdown of the molecule structure was observed after 30 days incubation as evidenced by the large number of minor unknowns which individually reached up to max. 6.8 % AR (after 30 days). The quantum yield for S-2200 *R*-isomer was determined to be 0.283.

Comments (RMS):

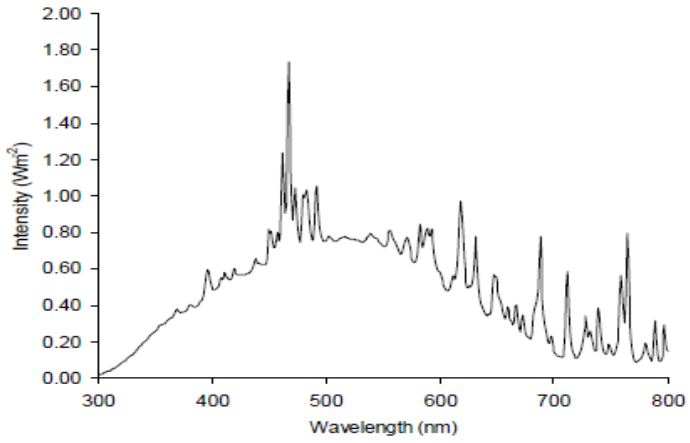
None.

Reference:	**[^{14}C]S-2354 (S-2200 *S*-isomer): Photodegradation and Quantum Yield in Sterile, Aqueous Solution.**
Author(s), year:	Lewis, C.J., Aldermann, D., 2010d
Study/report number:	8200195; ROM-0011
Guideline(s):	OECD 316 (2008), 95/36/EC, 94/37/EC, US-EPA N 161-2 (1982), J MAFF Nousan-8147 section 2-6-2 (2000)
GLP:	Yes
Deviations:	None
Validity:	Study considered acceptable

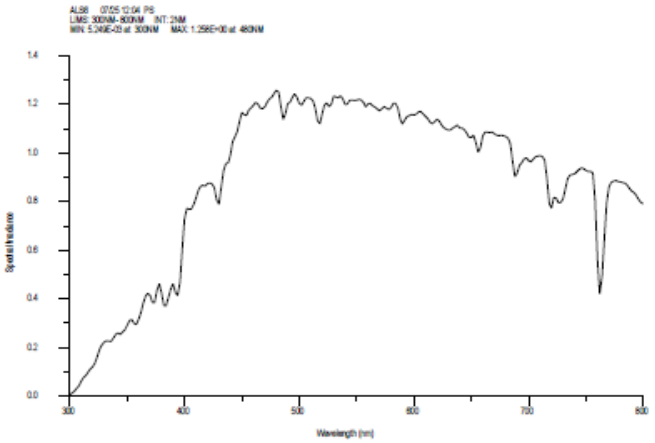
Material and methods:

Test substance:	[Benzyl-^{14}C]S-2354, 4.22 GBq mmol^{-1} (13.51 MBq mg^{-1}) ≥ 98 % radiochemical purity (HPLC), batch RIS2008-009, 100 % optical purity
Reference substances:	S-2354 (unlabelled), S-2167 (unlabelled, S-2200 R-isomer), 5-COOH-S-2200, 2-COOH-S-2200, S-2200-ORC, S-2200-OR, De-Xy-S-2200, DX-CA-S-2200, (all: purity > 99 %); S-2200-PR (purity: 89.6 %), MCBX (purity: 96.9 %)
Test system:	Sterile pH 7.0 buffer (0.01 M phosphate buffer, autoclaved), quartz glass units, sterility was checked throughout the experiment. A PNAP/PYR actinometer for determination of quantum yield.
Test temperature:	25 ± 1 °C
Test duration:	30 days continuous irradiation (30 day incubation equivalent to ca. 30 summer sunlight days in US and UK) or dark incubation.
Sample concentration:	1.0 mg L^{-1}
Co-solvent:	Acetonitrile
Test system:	Xenon lamp (Suntest Accelerated Exposure machine), cut-off < 290 nm, ca. 25 watts m^{-2} (300 – 400 nm).

Radiation Spectrum (300 to 800 nm) of the Xenon Lamp in Suntest Accelerated Exposure Machine 6 (position 4)



Radiation spectrum (300 to 800 nm) of Harrogate (*ca* 54°N) summer sunlight on 25 July 1995



Spectrum of experimental radiation is close to spectral distribution of natural sunlight (solar irradiation of Harrogate in summer).

Volatile traps:	Polyurethane foam bung, 1 x ethanediol trap and 2 x 2 M NaOH trap
Analysis:	LSC, HPLC-UV, 2D-TLC LOD < 1 % of AR (HPLC)
Kinetic evaluation:	Simple first order (SFO) kinetics, Microsoft Excel, KinGUI 1.1

Findings:

Sterile conditions were maintained throughout the study. Mass balance was in a range of 96.5 to 98.1 % of AR for all experiments. Unit rinses contained ≤ 2.5 % of AR and hence confirmed no adsorption of radioactivity to the glass vessels. The polyurethane foam bungs and ethanediol traps from the incubated samples contained max. 2.1 % of AR during the entire incubation period. The NaOH traps contained up to 2.1 % of AR after 30 days. Under irradiation S-2354 was subjected to extensive photolytic rearrangement procedures, resulting in two major metabolites: S-2200-OR (maximum 18.6 % of AR at DAT 7) and S-2200-ORC (maximum 10.5 % of AR at DAT 14 and at DAT 30). Other identified products were S-2200-PR (max. 7.5 % after 4 days) and De-Xy-S-2200 (max. 7.2 % after 30 days). S-2200-OR and S-2200-PR further degraded until the end of the study, whereas S-2200-ORC and De-Xy-S-2200 reached their maximum at the end of the study. In addition, trace amounts (≤3%) of 5-COOH-S-2200, MCBX and DX-CA-S-2200 were tentatively identified but could not be confirmed by TLC. A very large number of unknowns (up to 33) were formed which individually reached up to max. 8.1% AR.

Without irradiation no degradation of S-2354 was observed. At all sampling intervalls greater or equal to 93 % of AR was recovered as S-2354 and no degradation products were detected. No S-2167 was present in any sample (chiral HPLC) and therefore no isomerisation of S-2354 occurred. The results of HPLC analyses are shown in Table 85. The proposed photolysis degradation route of S-2354 is shown in Figure 2.

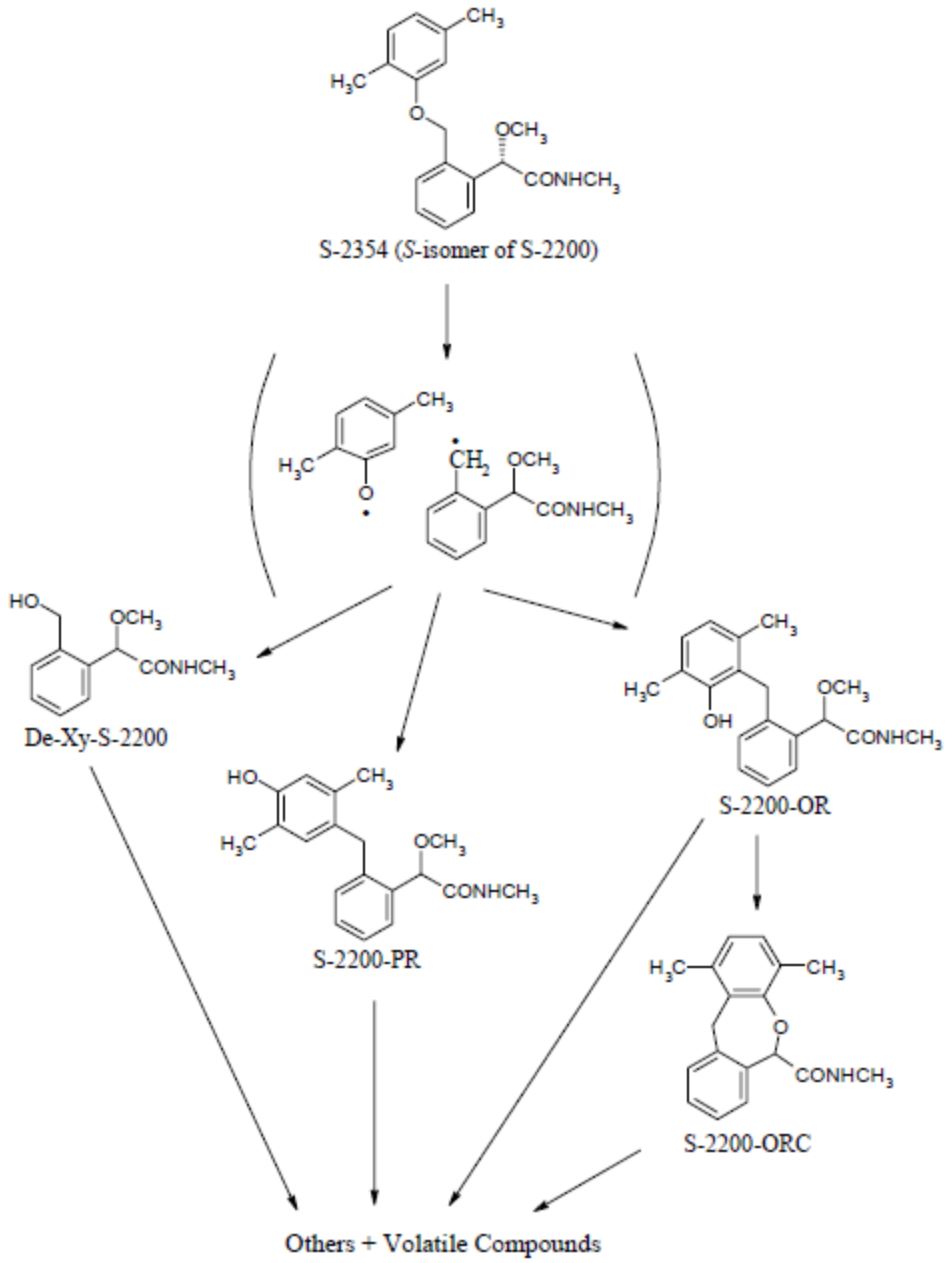


Figure 2: Proposed photolysis degradation route of S-2354 (S-2200 S-isomer)

Table 85: Photo-transformation of S-2354 in sterile water buffered at pH 7 [% of AR].

Label	Conditions	DAT	S-2354	S-2200-OR	S-2200-ORC	S-2200-PR	De-Xy-S-2200	Peak A*	Total unknowns (number)	Largest unknown	Organic volatiles	CO_2	Mass balance
[Benzyl-^{14}C]	Irradiated	0	93.4	ND	ND	ND	ND	ND	ND	ND	NA	NA	97.0
		1	80.7	7.4	0.1	4.0	ND	ND	1.6 (1)	1.9	2.1	ND	97.6
		2	73.8	10.0	0.6	5.7	0.4	ND	2.4 (2)	2.2	0.2	ND	97.1
		4	48.7	15.7	2.3	7.5	2.7	1.5	14.4 (18-29)	3.2	0.1	ND	98.1
		7	37.3	18.6	3.8	7.0	1.4	3.3	20.6 (22-27)	2.5	0.5	0.2	98.0
		14	6.5	9.7	10.5	2.4	3.2	6.3	50.4 (28-32)	7.2	1.1	0.7	96.5
		21	2.8	5.1	7.3	1.9	5.1	4.7	65.2 (32-33)	8.1	0.4	1.6	97.3
		30	2.7	3.9	10.5	1.1	7.2	5.2	56.5 (26-29)	5.9	0.3	2.1	97.9
	Dark	30	94.7	ND	ND	ND	ND	ND			0.2	ND	97.4

* consists of several peaks, each ≤ 2 % of AR
ND: not detected
NA: not applicable

Table 86: Calculated aquatic photolytic DT_{50} and DT_{90} [days] of S-2354 and metabolites using SFO kinetics at pH 7.

Compound	Label	DT_{50} (UK/US equivalent)	DT_{90} (UK/US equivalent)	Chi2-error
S-2354	Benzyl	4.6	15.3	5.63
S-2200-OR	Benzyl	4.0	13.2	10.97
S-2200-PR	Benzyl	2.2	7.1	10.99

The DT_{50} values determined for S-2354 are equivalent to US and UK summer sunlight. The first order DT_{50} value for S-2354 was 4.6 days. The DT_{50} values for the metabolites were determined by modelling. A mean SFO DT_{50} value of 4.0 days was calculated for S-2200-OR, whereas S-2200-ORC appeared stable to photolysis. The DT_{50} of S-2200-PR was 2.2 days.

The quantum yield of S-2354 was determined by direct comparison of the degradation rates of S-2354 and of PNAP in a binary actinometer, both exposed to identical lighting conditions. The quantum yield value for S-2354 was determined to be 0.269.

Conclusion:

Under irradiated experimental conditions, S-2354 degraded at a first order DT_{50} value which corresponds to about 4.6 days under environmental conditions in US/UK summer (reference Harrogate 54 °N). Appropriate controls confirmed that there was no degradation in darkness. No isomerisation from S-2200 *S*-isomer (S-2354) to S-2200 *R*-isomer (S-2167) occurred. The main photolytic metabolites were S-2200-OR (max. 18.6% after 7 days) and S-2200-ORC (max 10.5% after 14 and 30 days). A mean SFO DT_{50} value of 4.0 days was calculated for S-2200-OR, whereas S-2200-ORC appeared stable to photolysis. Other products identified were S-2200-PR (max. 7.5 % AR after 4 days) and De-Xy-S-2200 (max. 7.2 % AR after 30 days). The DT_{50} of S-2200-PR was calculated to be 2.2 days, the metabolite De-Xy-S-2200 reached its maximum at the end of the incubation period. Volatile radioactivity comprised < 3 % AR. Extensive breakdown of the molecule structure was observed after 30 days incubation as evidenced by the large number of unknowns which individually reached up to max. 8.1 % AR (21 days). The quantum yield for S-2200 *S*-isomer was determined to be 0.269.

Comments (RMS):

None.

5.1.2 Biodegradation

5.1.2.1 Biodegradation estimation

As measured data are available estimation is not relevant for this dossier.

5.1.2.2 Screening tests

Readily biodegradability:

Reference:	**S-2200 (Racemic Mixture): Assessment of Ready Biodegradability by Measurement of CO_2 evolution.**
Author(s), year:	Graham, R., 2009
Study/Report number:	1002463; ROM-0003
Guideline(s):	OECD 301 B Ready Biodegradability (Adopted 1981, Revised 1992)
GLP:	Yes
Deviations:	None
Validity:	Study considered acceptable

Material and methods:

Test substance:	S-2200 (unlabelled), purity 93.4 %, batch ST-0811G
Reference substance:	Sodium benzoate
Inoculum:	Return line of a waste water plant (Burley Menston) treating predominately domestic sewage (30 mg L^{-1})
Treatments:	Replicates (except for toxicity control): • Blank control • Reference substance: sodium benzoate (15 mg C L^{-1}) • Test substance: S-2200 (15 mgC L^{-1}) • Toxicity control: S-2200 (15 mgC L^{-1}) and sodium benzoate (15 mg C L^{-1}) pH of the test vessel at the end of test: 7.5 to 7.64
Analysis:	CO_2 amount absorbed by each trap calculated from the reduction in the concentration of barium hydroxide solution (titration)
Incubation conditions:	22 ± 2 °C, 28 days, in the dark

Findings:

Table 87: Biodegradation of S-2200 and reference compound [% of theoretically possible degradation]

DAT	Reference substance		Test substance	Toxicity control
	Replicate 1	Replicate 2		
1	12	12	0	8
3	46	47	0	40
6	59	59	0	54
8	65	65	0	61

DAT	Reference substance		Test substance	Toxicity control
	Replicate 1	Replicate 2		
10	69	69	0	65
14	76	76	0	75
17	81	82	0	81
20	85	86	1	85
23	87	88	1	87
28	89	90	1	89
29	90	92	2	90

Within 28 days almost no degradation (maximum 2 %) was determined for S-2200. The reference substance (sodium benzoate) has reached level for ready biodegradability by 8 days. The toxicity control showed values similar to the values of the reference substance indicating that S-2200 is not toxic to the microorganisms of the activated sludge under test conditions.

Conclusion:

S-2200 is considered to be not readily biodegradable.

Comments (RMS):

None

5.1.2.3 Simulation tests

Biodegradation in water/sediment systems:

Two water sediment studies, one study with each isomer, were conducted:

- S-2167 (S-2200 *R*-isomer): Aerobic water/sediment study with two test systems, [benzyl-^{14}C] and [phenoxy-^{14}C] label
- S-2354 (S-2200 *S*-isomer): Aerobic water/sediment study with two test systems, [benzyl-^{14}C] label

Reference:	**[^{14}C]S-2167 (S-2200 *R*-isomer): Degradation in Water-Sediment Systems under Aerobic Conditions**
Author(s), year:	Graham, R., 2011a
Study/report number:	8200193, ROM-0022
Guideline(s):	OECD Guideline 308 (2002)
GLP:	Yes
Deviations:	Minor
Validity:	Study considered acceptable

Material and methods:

Test substances: S-2167, S-2200 *R*-isomer
(*R*)-2-[2-(2,5-dimethylphenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamid

- [Benzyl-^{14}C]S-2167 (S-2200 *R*-isomer) (Batch number: RIS2008-010); specific radioactivity 13.48 MBq mg^{-1} (4.22 GBq mmol^{-1}), Radiochemical purity: 99.2; chemical purity: 98 % (HPLC, UV

	detection at 254 nm), optical purity: 100% (HPLC, UV detection at 275 nm). • [Phenoxy-^{14}C]S-2167 (S-2200 *R*-isomer) (Batch number: RIS2009-002); specific radioactivity 14.16 MBq mg^{-1} (4.44 GBq mmol^{-1}), Radiochemical purity: 99.1%; chemical purity: 98.3 % (HPLC, UV detection at 254 nm), optical purity: 100% (HPLC, UV detection at 275 nm). • Non-labelled S-2167 (Batch number: 060020652); chemical purity: 100 %.
Reference substances:	Non-labelled S-2354 (S-2200 *S*-isomer), 2-COOH-S-2200, 5-COOH-S-2200, De-Xy-S-2200, DX-CA-S-2200, (*S*)-MCBX, 2,5-dimethylphenol (2,5-DMP), chemical purity: > 99 %; MCBX, chemical purity: 96.9 %, (*R*)-MCBX, chemical purity: 98.2 %
Application rate:	2.9 µg/unit (water surface area of 15.9 cm^2, 9 cm water depth), equivalent to ca. 200 g ai/ha
Incubation set-up:	3 cm depth of dry Calwich Abbey or Swiss Lake sediment (2 mm sieved) in individual borosilicate glass cylinder (ca 4.5 cm in diameter), 9 cm depth of associated water (0.2 mm sieved). Sediment units were aerated, slightly agitated on an orbital shaker. Flow through system (moistured air at flow rate of ca. 20-60 mL min^{-1}).
Acclimatization period:	27 days
Test duration:	101 days
Incubation conditions:	20 ± 2 °C in darkness
Sampling:	0, 7, 14, 31, 62, 101 days
Volatile traps:	Ethanediol (polar volatiles), 2 % paraffin in xylene (non-polar volatiles), two 2 M sodium hydroxide (CO_2, confirmation of CO_2 not required since CO_2 < 5 % AR in all samples)
Analysis:	Water samples partitioned three to four times with ethyl-acetate, organic fraction combined and radio-assayed by LSC. Sub-samples of the organic fraction concentrated prior to LSC and chromatograpic analysis (HPLC). Sub-samples of the aequous fraction of two units containing > 5 % AR concentrated and analysed by LSC and HPLC. The identities of metabolites in the extracts were confirmed by TLC of selected samples. Sediment phase extracted twice with 100 mL of acetone: water (9:1 v/v) and once with 100 mL aceton. The extracts (primary extract, neutral extract) were combined, sub-samples concentrated and quantified by LSC and analysed chromatographically. Sediments were further extracted twice with acetone : 0.1 M hydrochloric acid (5:1, v/v, 2 x 100 ml) and once with acetone (1 x 100 ml). The extracts (secondary extract, acidic extract) were combined, sub-samples concentrated and quantified by LSC and analysed chromatographically. The identities of metabolites in the extracts were confirmed by TLC of selected samples. Remaining sediment residues were dried, ground for combustion and analysed by LSC. Trapping solutions: radioactivity quantified by LSC. Selected samples were analysed to determine the optical purtity of S-2167. An HPLC Chiral-Pak AD-RH column was used with isocratic elution of a

	mobile phase containing 1:1 v/v Acetonitrile:water. Water, neutral sediment extracts and acidic sediment extracts were analysed.
Analytical techniques:	LSC (LOD ca 0.1 % of AR), HPLC (LOD ca 0.1 % of AR), TLC
Kinetic evaluation:	Simple first order (SFO) kinetics, first order multi-compartment (FOMC), double first-order in parallel (DFOP), KinGui v. 1.1

Table 88: Physicochemical characteristics of the water/sediment matrices.

Name		**Calwich Abbey**	**Swiss Lake**
Geographic location		Calwich Abbey lake, Calwich, Ashbourne, Derbyshire, UK	Swiss Lake, Chatsworth, Derbyshire, UK
Sediment	Texture (USDA)	Silt loam	Loamy Sand
	Sand (USDA) [%]	29	84
	Silt (USDA) [%]	64	13
	Clay (USDA) [%]	7	3
	pH ($CaCL_2$)	7.5	5.1
	pH (1 M KCl)	7.6	5.0
	pH (water)	8.0	5.9
	Organic C [%]	5.0	0.9
	Organic Matter [%]	8.6	1.6
	Redox [mV]	-137	-242
	CEC [mEq 100 g^{-1}]	17.9	4.6
	Microbial Biomass [µg C g^{-1}] – Start / End	1561 / 2754	356 / 704
Water	pH (water, sampling)	7.7	6.7
	Water hardness [mg L^{-1} as $CaCO_3$]	250	25
	Oxygen content [mg L^{-1}]	7	7
	Conductivity [µS cm^{-1}]	324	44
	Redox [mV] – Start	392	414
	TOC [ppm] – Start / End	4.5 / 8.2-8.6	10.4 / 6.0-7.7
	Suspended solids [mg L^{-1}]	51.4	33.2

Findings:

The oxygen content of the water phase was maintained >= 7 mg L^{-1} throughout the incubation period in both systems. This indicates aerobic conditions throughout the experiments. In the Calwich Abbey water sediment system the pH in the water phase remained relatively constant (decreased from pH 8.5 at the start of the study to pH 8.0 at the end) as well as the pH in the sediment system (rose slightly from pH 7.1 at the start to pH 7.4 at the end). In the Swiss Lake water sediment system the pH in the water phase and the pH in the sediment phase showed comparable values at the start (7.4 and 6.5) and at the end (7.5 and 6.8) of the study, but both values showed a decline 31 (6.8 and 6.2) and 60 (6.1 and 5.9) DAT. The water redox potential in the Calwich Abbey water sediment system remained relatively constant showing values between 370 to 420 mV. The water redox potential in the Swiss Lake System was about 400 mV at the start and at the end of the study, during the study the values increased to about 520 mV. In the sediment of Calwich Abbey the redox potential was < -100 mV for most of the incubation period, in the sediment of Swiss Lake the redox potential remained < -100 mV during the incubation period.

Total mass balance was in a range of 97 to 101 % of AR for both systems and both labels.

Distribution and recovery of radioactivity in both water/sediment systems are presented in table 89 and 90. Only data on major fractions (>5 % AR) are shown. Therefore, the mass balance values presented in the tables below do not fit the presented data.

Formation of $^{14}CO_2$ using [benzyl-^{14}C] label accounted for maximum 1.4 % of AR in the Calwich Abbey system and for maximum 3.7 % of AR in the Swiss lake system at study termination. Formation of $^{14}CO_2$ using [phenoxy-^{14}C] label accounted for maximum 2.4 % of AR in the Calwich Abbey system and 2.3 % of AR in the Swiss lake system. In all incubations there was no radioactivity in the traps for volatile organic compounds.

Formation of NER increased up to maximum 7.9 % of AR in the Calwich Abbey system and up to 5.8 % AR in the Swiss lake system with [benzyl-^{14}C] label. Using [phenoxy-^{14}C] label the formation of NER increased up to maximum 7.8 % of AR in the Calwich Abbey system and up to 5.1 % of AR in the Swiss lake system.

Table 89: Distribution and recovery of radioactivity [% of AR] after application of [benzyl-14C] labelled S-2167 (200 g ai ha-1) to the aerobic water/sediment systems ‘Calwich abbey’ and ‘Swiss lake’.

System	Time	Water			Sediment			Unextractable	CO_2	Mass balance	Total water/sediment		
	(day)	S-2167	2-COOH-S-2200	5-COOH-S-2200	S-2167	2-COOH-S-2200	5-COOH-S2200				S-2167	2-COOH-S-2200	5-COOH-S-2200
Calwich abbey	0*	94.0	ND	ND	4.2*	NA	NA	0.2	NA	98.8	98.2*	ND	ND
	7	50.8	ND	ND	44.4	ND	ND	1.0	0.1	98.3	95.2	ND	ND
	14	37.1	ND	0.2	54.7	ND	0.4	1.6	0.1	98.6	91.8	ND	0.7
	31	20.4	ND	0.1	68.5	ND	0.5	2.4	0.4	97.4	89.0	ND	0.6
	62	11.1	ND	ND	73.2	0.4	1.1	5.8	0.8	100.2	84.3	0.4	1.1
	101	8.6	0.7	2.8	70.8	ND	1.5	7.9	1.4	99.9	79.4	0.7	4.3
Swiss lake	0*	96.7	ND	ND	2.8*	NA	NA	ND	NA	100.2	99.5*	ND	ND
	7	60.7	ND	ND	33.9	ND	ND	0.4	0.2	98.8	94.6	ND	ND
	14	51.5	ND	ND	40.2	ND	0.1	0.9	0.2	97.7	91.7	ND	0.1
	31	38.1	0.5	1.2	48.0	0.2	0.4	1.4	0.7	97.3	86.1	0.6	1.6
	62**	22.5	0.4	1.4	56.5/4.6**	0.2	1.1/0.3**	4.1	2.6	99.6	83.7	0.6	2.8
	101**	18.1	1.4	3.1	55.5/6.6**	0.4	1.1/0.2**	5.8	3.7	99.7	80.2	1.9	4.4

NA…not applicable
ND…not detected
*assuming all radioactivity in neutral extract was S-2167
**sediment values: neutral/acidic extract (only analysed if >5 %AR)

Table 90: Distribution and recovery of radioactivity [% of AR] after application of [phenoxy-14C] labelled S-2167 (200 g ai ha-1) to the aerobic water/sediment systems ‘Calwich abbey’ and ‘Swiss lake’.

System	Time (day)	Water			Sediment			Unextractable	CO_2	Mass balance	Total water/sediment		
		S-2167	2-COOH-S-2200	5-COOH-S-2200	S-2167	2-COOH-S-2200	5-COOH-S2200				S-2167	2-COOH-S-2200	5-COOH-S-2200
Calwich abbey	0*	94.6	ND	ND	2.2*	NA	NA	0.1	NA	97.7	96.8*	ND	ND
	7	45.4	ND	ND	49.1	ND	0.3	1.3	0.2	99.0	94.5	ND	0.3
	14	34.6	ND	ND	58.4	ND	ND	1.2	0.1	97.7	93.0	ND	ND

System	Time (day)	Water			Sediment			Unextractable	CO_2	Mass balance	Total water/sediment		
		S-2167	2-COOH-S-2200	5-COOH-S-2200	S-2167	2-COOH-S-2200	5-COOH-S2200				S-2167	2-COOH-S-2200	5-COOH-S-2200
	31	13.7	ND	0.1	72.9	ND	1.1	3.0	0.7	98.1	86.6	ND	1.2
	62	7.7	ND	2.3	67.6	ND	4.3	6.9	1.5	99.7	75.3	ND	6.6
	101	7.1	ND	0.3	72.1	ND	0.8	7.8	2.4	98.0	79.2	ND	1.1
Swiss lake	0*	97.4	ND	ND	1.3*	NA	NA	ND	NA	99.1	98.7*	ND	ND
	7	63.1	ND	ND	32.1	ND	ND	0.3	0.2	97.4	95.2	ND	ND
	14	53.2	ND	ND	39.5	ND	ND	1.5	0.3	98.4	92.6	ND	ND
	31	39.5	0.2	1.0	51.1	ND	0.2	0.8	0.8	99.9	90.6	0.2	1.2
	62**	26.2	0.8	2.1	59.3/5.6**	0.1	0.9/0.3**	3.2	1.2	100.5	91.1	0.9	3.3
	101**	19.2	0.9	1.4	60.3/7.0**	0.4	1.5/0.1**	5.1	2.3	98.9	86.4	1.3	3.0

NA…not applicable
ND…not detected
*assuming all radioactivity in neutral extract was S-2167
**sediment values: neutral/acidic extract (only analysed if >5 %AR)

S-2167 degraded in both water/sediment systems. Transfer of the test substance into sediment was relatively fast with occurrences of S-2167 between 32 % AR and 49 % AR after 7 days. Maximum occurrence of [benzyl-^{14}C] labelled S-2167 in sediment was 73.2 % AR (DAT 62) and 62.1 % AR (DAT 101) in Calwich Abbey and Swiss Lake respectively. For [phenoxy-^{14}C] labelled S-2167 the maximum occurrence in sediment was 72.9 % of AR (DAT 31) and 67.3 % of AR (DAT 101) in Calwich Abbey and Swiss Lake respectively. No notable degradation of S-2167 was observed in the sediment for any of the treated groups over the duration of the study. No S-2354 (*S*-isomer) was detected in representative samples analysed for optical purity indicating that no isomerisation of S-2167 occurred (co-elution with the tested isomer S-2167 occurred and there was no evidence of any ^{14}C co-eluting with *S*-isomer S-3554).

No metabolites were present in either water sediment system at levels > 5 % AR in two consecutive timepoints or > 10 % AR at any timepoint. The metabolite 2-COOH-S-2200 was detected in surface water and sediment and accounted for a maximum of 1.9 % AR at 101 DAT in the [benzyl-^{14}C]S-2167 treated Swiss Lake system. The metabolite 5-COOH-S-2200 was detected in both water and sediment accounting for a maximum of 6.6 % AR at 62 DAT in the [phenoxy-14C]S-2167 treated Calwich Abbey system. MCBX was present at < 1 % AR in water and sediment. All unknown metabolites accounted for less than 2 % AR in water and/or sediment.

The proposed degradation pathway of S-2167 in water/sediment system is shown in figure 3 below. Degradation of S-2167 occurred by oxidation of the methyl group at the 2- and 5-position of the phenoxy ring system generating the metabolites 2-COOH-S-2167 and 5-COOH-S-2167. Further oxidation of the other methyl group (at the 2- and 5-position of the phenoxy ring system) and *O*-demethylation of the benzyl ring system side-chain resulted in formation of the metabolite MCBX.

The degradation rates were fitted with Single First Order (SFO) kinetic. In addition First Order Multi-Compartment (FOMC) and Double First Order in Parallel (DFOP) kinetic was also presented. The most appropriate model was selected depending on the best fit, based on visual inspection and assessment of the Chi2 values.

In the total water sediment system SFO kinetics was considered acceptable for determining simulation endpoints allowing good visual fit and χ^2 error values below 4 %. The optimised parameters and associated statistics for S-2167 are presented in Table 91.

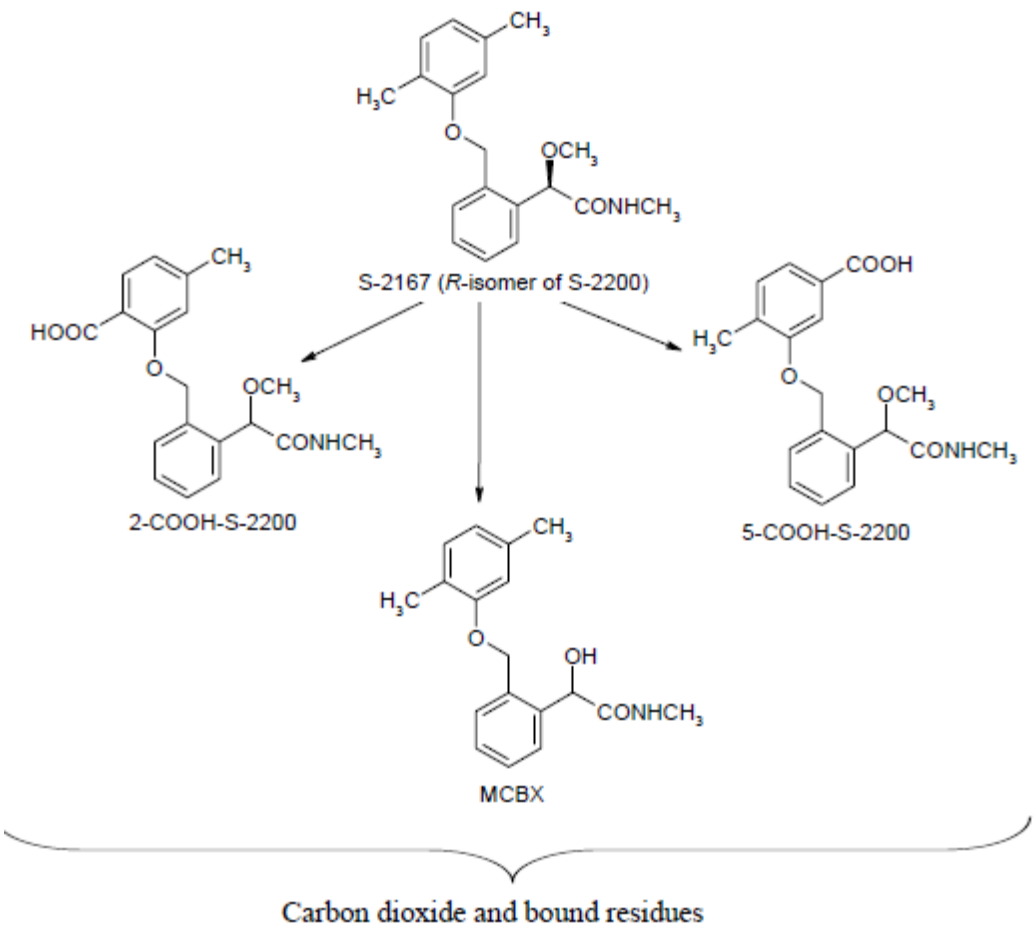


Figure 3: Proposed metabolic pathway for S-2200 in water-sediment systems

Table 91: Degradation rates of S-2167 in water sediment systems (whole system) following Single First Order (SFO) kinetics and First Order Multi-Compartment (FOMC) kinetics and Double First Order in Paralell (DFOP) kinetics.

Parameter	Calwich Abbey		Swiss Lake	
	[Benzyl-^{14}C]	**[Phenoxy-^{14}C]**	**[Benzyl-^{14}C]**	**[Phenoxy-^{14}C]**
Model	**SFO**	**SFO**	**SFO**	**SFO**
Chi^2 error [%]	**1.14**	**3.15**	**2.2**	**1.47**
DT_{50} [Day]	**342** (1×10^{-6})	**284** (3×10^{-4})	**344** (1.3×10^{-5})	**654** (1.2×10^{-4})
DT_{90} [Day]	**>1000**	**942**	**>1000**	**>1000**
Model	DFOP	FOMC	DFOP	DFOP
Chi^2 error [%]	1.41	2.63	2.66	1.8
DT_{50} [Day]	355	>1000	463	>1000
DT_{90} [Day]	>1000	>1000	>1000	>1000

*P value from the t-test is given in brackets.

For dissipation in the surface water the FOMC model gave the best fit kinetics with good visual fit and χ^2 error values below 6 %. The optimised parameters and associated statistics for S-2167 are presented in Table 92.

Table 92: Degradation rates of S-2167 in water sediment systems (surface water) following Single First Order (SFO) kinetics and First Order Multi-Compartment (FOMC) kinetics.

Parameter	Calwich Abbey		Swiss Lake	
	[Benzyl-^{14}C]	**[Phenoxy-^{14}C]**	**[Benzyl-^{14}C]**	**[Phenoxy-^{14}C]**
Model	SFO	SFO	SFO	SFO
Chi^2 error [%]	14.67	13.93	14.68	14.06
DT_{50} [Day]	12	9	28	31
DT_{90} [Day]	39	30	93	104
Model	**FOMC**	**FOMC**	**FOMC**	**FOMC**
Chi^2 error [%]	**1.87**	**5.66**	**3.71**	**2.73**
DT_{50} [Day]	**9**	**7**	**15**	**17**
DT_{90} [Day]	**83**	**56**	**333**	**416**

Conclusion:

In both water/sediment systems and for both labels S-2167 degraded via the metabolites 2-COOH-S-2200 and 5-COOH-S-2200 which reached maximum levels of 1.9 % AR and 6.6 % AR in the whole system, respectively. Further degradation led to the metabolite MCBX which accounted for < 1 % AR in water and sediment. No other compounds were detected at levels above 2 % of AR in water and / or sediment. Mineralisation over 101 days was relatively small (1.4 – 3.7 % of AR) and not greatly different between the [benzyl-^{14}C] and [phenoxy-^{14}C] labelled forms of S-2167 and between the two water/sediment systems. Amounts of bound residues remaining after 101 days were also very similar between the two radiolabelled forms and between the two water/sediment systems being 5.1 – 7.9 % of AR. S-2167 dissipated from the water and reached maximum amounts of 73 % of AR in the sediment phase (31 and 62 DAT, Calwich Abbey system). Degradation in the whole system was determined using SFO (following guidance document FOCUS, 2006) with first order DT_{50} values of 342 and 344 days for the [benzyl-^{14}C] labelled S-2167 and with first order DT_{50} values of 284 and 654 days for the [phenoxy-^{14}C] labelled S-2167. The respective DT_{90} values were in the range of 942 - >1000 days.

Comments (RMS):

The study is considered correct.

Reference:	**[^{14}C]S-2354 (S-2200 *S*-isomer): Degradation in Water-Sediment Systems under Aerobic Conditions**
Author(s), year:	Graham, R., 2011b
Study/report number:	8200200, ROM-0023
Guideline(s):	OECD Guideline 308 (2002)
GLP:	Yes
Deviations:	Minor
Validity:	Study considered acceptable

Material and methods:

Test substances:	S-2354, S-2200 *S*-isomer (*S*)-2-[2-(2,5-dimethylphenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamid • [Benzyl-^{14}C]S-2354 (S-2200 *S*-isomer) (Batch number: RIS2008-009); specific radioactivity 13.51 MBq mg^{-1} (4.22 GBq mmol^{-1}), Radiochemical purity: 99.4; chemical purity: 98 % (HPLC, UV detection at 254 nm), optical purity: 100% (HPLC, UV detection at 275 nm). • Non-labelled S-2354 (Batch number: 0600206523; chemical purity: 99.7 %.
Reference substances:	Non-labelled S-2167 (S-2200 *R*-isomer), 2-COOH-S-2200, 5-COOH-S-2200, De-Xy-S-2200, DX-CA-S-2200, (*S*)-MCBX; MCBX, chemical purity: 96.9 %, (*R*)-MCBX, chemical purity: 98.2 %
Application rate:	2.9 µg/unit (water surface area of 15.9 cm^2, 9 cm water depth), equivalent to ca. 200 g ai/ha
Incubation set-up:	3 cm depth of dry Calwich Abbey or Swiss Lake sediment (2 mm sieved) in individual borosilicate glass cylinder (ca 4.5 cm in diameter), 9 cm depth of associated water (0.2 mm sieved). Sediment units were aerated, slightly agitated on an orbital shaker. Flow through system (moistured air at flow rate of ca. 20-60 mL min^{-1}).
Acclimatization period:	23 days
Test duration:	102 days
Incubation conditions:	20 ± 2 °C in darkness
Sampling:	0, 7 or 8, 14, 29, 60, 102 days
Volatile traps:	Ethanediol (polar volatiles), 2 % paraffin in xylene (non-polar volatiles), two 2 M sodium hydroxide (CO_2; confirmation of CO_2 not required since level of radioactivity < 5 % AR in all traps)
Analysis:	Water samples partitioned three times with ethyl-acetate, organic fraction combined and radio-assayed by LSC. Sub-samples of the organic fraction concentrated prior to LSC and chromatograpic analysis (HPLC). The identities of metabolites in the extracts were confirmed by TLC of selected samples. Sediment phase extracted twice with 100 mL of acetone: water (9:1 v/v) and once with 100 mL aceton. The extracts (primary extract, neutral extract) were combined, sub-samples concentrated and quantified by LSC and analysed chromatographically (HPLC). Sediments were further extracted twice with acetone : 0.1 M hydrochloric acid (5:1, v/v, 2 x 100 ml) and once with acetone (1 x 100 ml). The extracts (secondary extract, acidic extract) were combined, sub-samples concentrated and quantified by LSC and analysed chromatographically (HPLC). The identities of metabolites in the extracts were confirmed by TLC of selected samples. Remaining sediment residues were dried, ground for combustion and analysed by LSC. Trapping solutions: radioactivity quantified by LSC. Selected samples were analysed by chiral HPLC to determine the optical putity of S-2354 and the optical isomer ratio of the metabolite MCBX. For parent compound an HPLC Chiral-Pak AD-RH column was used with

	isocratic elution of a mobile phase containing 1:1 v/v Acetonitrile:water. Water, neutral sediment extracts and acidic sediment extracts were analysed. For MCBX an HPLC Chiral-Pak AD-RH column was used with isocratic elution of a mobile phase containing 0.35:0.65 v/v Acetonitrile:water. Water and neutral sediment extracts from the final timepoint at Calwich Abbey were analysed.
Analytical techniques:	LSC (LOD ca 0.1 % of AR), HPLC (LOD ca 0.1 % of AR), TLC
Kinetic evaluation:	Simple first order (SFO) kinetics, first order multi-compartment (FOMC), double first-order in parallel (DFOP), KinGui v. 1.1

Table 93: Physicochemical characteristics of the water/sediment matrices.

Name		**Calwich Abbey**	**Swiss Lake**
Geographic location		Calwich Abbey lake, Calwich, Ashbourne, Derbyshire, UK	Swiss Lake, Chatsworth, Derbyshire, UK
Sediment	Texture (USDA)	Silt loam	Loamy Sand
	Sand (USDA) [%]	29	84
	Silt (USDA) [%]	64	13
	Clay (USDA) [%]	7	3
	pH ($CaCL_2$)	7.5	5.1
	pH (1 M KCl)	7.6	5.0
	pH (water)	8.0	5.9
	Organic C [%]	5.0	0.9
	Organic Matter [%]	8.6	1.6
	Redox [mV] . Start / End	-200 / -108	-188 / 86
	CEC [mEq 100 g^{-1}]	17.9	4.6
	Microbial Biomass [µg C g^{-1}] – Start / End	1547 / 2138	309 / 517
Water	pH (water, sampling)	7.7	6.7
	Water hardness [mg L^{-1} as $CaCO_3$]	250	25
	Oxygen content [%]	7	8
	Conductivity [µS cm^{-1}]	324	44
	Redox [mV] – Start / End	418 / 418	393 / 662
	TOC [ppm] – Start	4.5	10.4
	Suspended solids [mg L^{-1}]	51.4	33.2

Findings:

The mean oxygen content of the water phase was maintained >= 7 mg L^{-1} throughout the incubation period in both systems. This indicates aerobic conditions throughout the experiments. In the Calwich Abbey water sediment system the pH in the water phase remained relatively constant (increased from pH 7.6 at the start of the study to pH 7.7 at the end) as well as the pH in the sediment system (increased from pH 6.8 at the start to pH 7.3 at the end). In the Swiss Lake water sediment system the pH in the water phase and the pH in the sediment phase slightly increased from the start until 29 DAT and decreased notably 60 and 102 DAT. The water redox potential in the Calwich Abbey water sediment system remained relatively constant showing values about 400 mV. The water redox potential in the Swiss Lake System was about 400 mV at the start of the study, and increased to about 662 mV until the end of the study. In the sediment of

Calwich Abbey the redox potential was < -100 mV for most of the incubation period, in the sediment of Swiss Lake the redox potential remained < -100 mV during the incubation period with the exception of one replicate at 60 DAT and 102 DAT.

Total mass balance was in a range of 97 to 100 % of AR. Distribution and recovery of radioactivity in both water/sediment systems are presented in table 94. Only data on major fractions (>5 % AR) are shown. Therefore, the mass balance values presented in the tables below do not fit the presented data.

Formation of $^{14}CO_2$ using [benzyl-^{14}C] label accounted for maximum 1.3 % of AR in the Calwich Abbey system and for maximum 1.2 % of AR in the Swiss lake system at study termination. In all incubations there was no radioactivity in the traps for volatile organic compounds.

Formation of NER increased up to maximum 9.1 % of AR in the Calwich Abbey system and up to 6.9 % AR in the Swiss lake system with [benzyl-^{14}C] label.

Table 94: Distribution and recovery of radioactivity [% of AR] after application of [benzyl-14C] labelled S-2354 (200 g ai ha-1) to the aerobic water/sediment systems ‘Calwich abbey’ and ‘Swiss lake’.

System	Time	Water				Sediment				Un-extractable	CO_2	Mass balance	Total water/sediment			
	(day)	S-2354	2-COOH-S-2200	5-COOH-S-2200	MCBX	S-2354	2-COOH-S-2200	5-COOH-S-2200	MCBX				S-2354	2-COOH-S-2200	5-COOH-S-2200	MCBX
Calwich abbey	0*	94.7	ND	ND	ND	2.5*	NA	NA	NA	ND	NA	97.4	97.2*	ND	ND	ND
	7	47.5	ND	ND	ND	46.7	ND	ND	1.0	1.0	0.1	98.6	94.2	ND	ND	1.0
	14	32.8	ND	ND	0.3	59.6	ND	ND	1.0	1.4	0.1	98.2	92.3	ND	ND	1.3
	29	18.3	ND	ND	0.6	68.6	ND	ND	3.5	3.1	0.2	98.9	86.9	ND	ND	4.1
	60	7.9	ND	ND	1.4	61.3	ND	0.6	15.0	6.8	0.6	99.6	69.2	ND	0.6	16.4
	102**	7.6	ND	0.3	2.7	53.6/3.5**	ND	0.5	14.5/0.9**	9.1	1.3	97.8	64.7	ND	0.9	18.0
Swiss lake	0*	92.4	ND	ND	ND	3.9*	NA	NA	NA	ND	NA	96.6	96.3*	ND	ND	ND
	7	59.2	ND	ND	ND	35.0	ND	ND	ND	1.1	0.1	97.9	94.2	ND	ND	ND
	14	52.7	ND	ND	ND	41.0	ND	ND	0.3	0.6	0.1	98.0	93.7	ND	ND	0.3
	29	42.0	ND	ND	0.3	46.6	ND	ND	1.2	1.5	0.2	97.9	88.6	ND	ND	1.5
	60**	28.5	0.5	1.3	0.9	54.9/3.3**	ND	0.4	2.9	2.2	0.8	98.7	86.7	0.5	1.7	3.8
	102**	20.7	0.1	0.3	0.3	57.5/7.5**	ND	0.2	2.6/0.2**	6.9	1.2	98.3	85.8	0.1	0.5	3.1

NA…not applicable
ND…not detected
*assuming all radioactivity in neutral extract was S-2354
**sediment values: primary-neutral/secondary-acidic extract (only analysed if >5 %AR)

S-2354 degraded in both water/sediment systems showing a faster degradation in Calwich Abbey system than in Swiss lake system. Transfer of the test substance into sediment was relatively fast with occurrences of S-2354 between 35 % AR and 47 % AR after 7 days. Maximum occurrence of [benzyl-^{14}C] labelled S-2354 in sediment was 69 % AR (DAT 29) and 65 % AR (DAT 102) in Calwich Abbey and Swiss Lake respectively. No notable degradation of S-2354 was observed in the sediment of Swiss Lake system over the duration of the study. In Calwich Abbey the amount of S-2354 decreased from its peak of 69 % AR on 29 DAT to 57 % AR until the end of the study. No S-2167 (*R*-isomer) was detected in representative samples analysed for optical purity indicating that no isomerisation of S-2354 occurred (co-elution with the tested isomer S-2354 occurred and there was no evidence of any ^{14}C co-eluting with *R*-isomer S-2167).

Only one metabolite, MCBX, was present at levels > 5 % AR in two consecutive timepoints or > 10 % AR at any timepoint. In Calwich Abbey MCBX reached up to 18 % AR in the whole system (102 DAT) and up to 15.4 % AR in sediment. In surface water of both systems and in sediment of Swiss lake system MCBX accounted for less than 5 % AR. Only the S-isomer of MCBX was detected (co-elution with the *S*-isomer of MCBX was seen). The metabolite 2-COOH-S-2200 was detected in the Swiss lake surface water only and accounted for <1 % AR. The metabolite 5-COOH-S-2200 was detected in both water and sediment accounting for a maximum of 1.7 % AR at 60 DAT in the Swiss lake whole system. No unknown metabolites were detected in Calwich Abbey system and one unknown metabolite was detected in Swiss lake sediment only, accounted for less than 2 % AR.

The proposed degradation pathway of S-2354 in water/sediment system is shown in figure 4 below. Degradation of S-2354 occurred by oxidation of the methyl group at the 2- and 5-position of the phenoxy ring system generating the metabolites 2-COOH-S-2200 and 5-COOH-S-2200. Further oxidation of the other methyl group (at the 2- and 5-position of the phenoxy ring system) and *O*-demethylation of the benzyl ring system side-chain resulted in formation of the metabolite MCBX.

The degradation rates were fitted with Single First Order (SFO) kinetic. In addition First Order Multi-Compartment (FOMC) and Double First Order in Parallel (DFOP) kinetic was also presented. The most appropriate model was selected depending on the best fit, based on visual inspection and assessment of the Chi^2 values.

In the total water sediment sysem SFO kinetics was considered acceptable for determining simulation endpoints allowing good visual fit and χ^2 error values below 3 %. The optimised parameters and associated statistics for S-2354 are presented in Table 95.

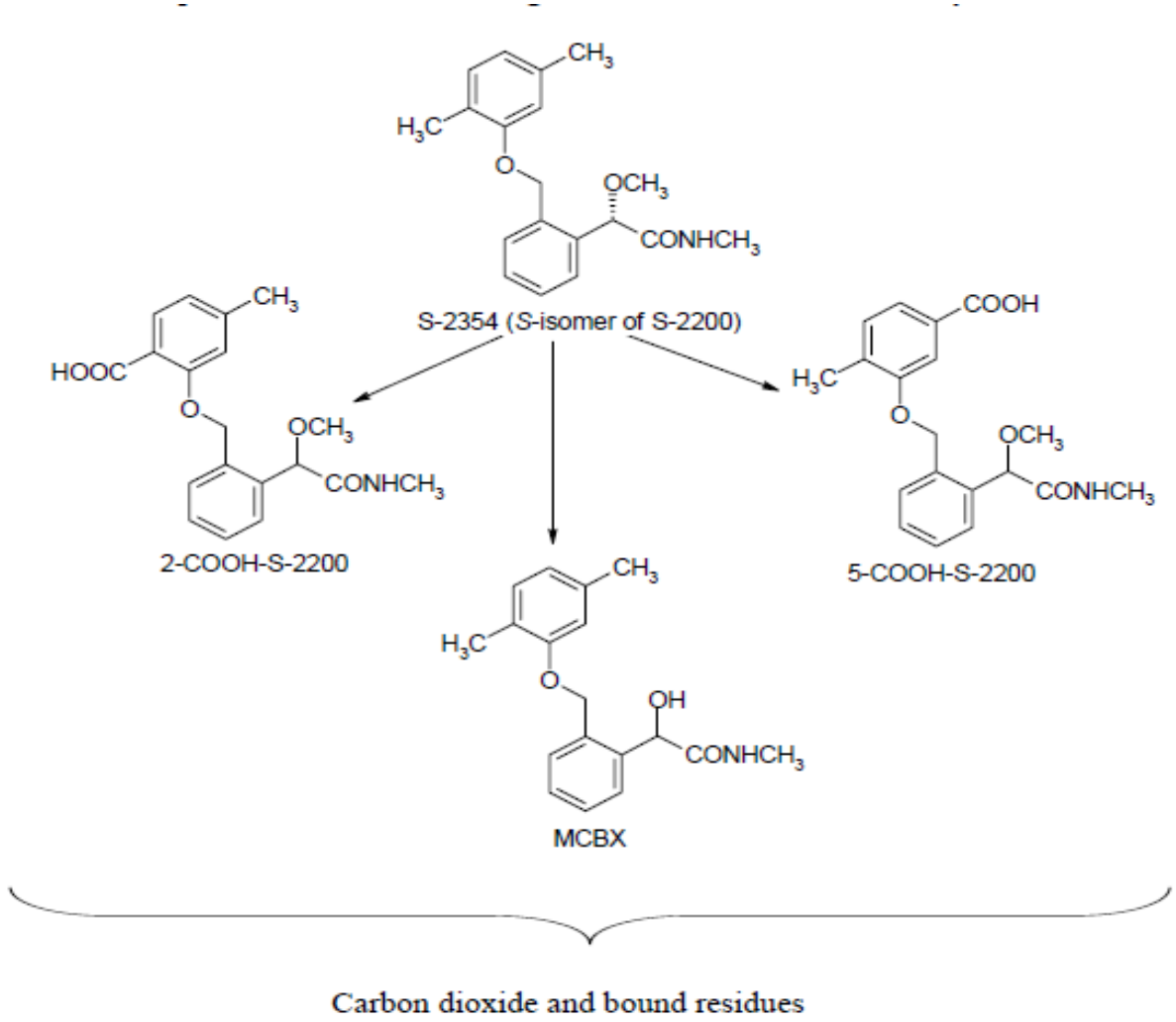


Figure 4: Proposed metabolic pathway for S-2354 in water-sediment systems

Table 95: Degradation rates of S-2354 in water sediment systems (whole system) following Single First Order (SFO) kinetics

Parameter	Calwich Abbey	Swiss Lake
	[Benzyl-^{14}C]	**[Benzyl-^{14}C]**
Model	**SFO**	**SFO**
Chi^2 error [%]	**2.3**	**1.6**
DT_{50} [Day]	**155** (7.9×10^{-8})	**600** (2.3×10^{-4})
DT_{90} [Day]	**516**	**>1000**

*P value from the t-test is given in brackets.

For dissipation in the surface water the FOMC model or DFOP model gave the best fit kinetics with good visual fit and χ^2 error values below 4 %. The optimised parameters and associated statistics for S-2354 are presented in Table 96.

Table 96: Degradation rates of S-2354 in water sediment systems (surface water) following Single First Order (SFO) kinetics and First Order Multi-Compartment (FOMC) and Double First Order in Paralell (DFOP) kinetics.

Parameter	**Calwich Abbey**	**Swiss Lake**
	[Benzyl-^{14}C]	**[Benzyl-^{14}C]**
Model	SFO	SFO
Chi^2 error [%]	13.3	14.1
DT_{50} [Day]	10	37
DT_{90} [Day]	32	121
Model	**FOMC**	**DFOP**
Chi^2 error [%]	**3.5**	**3.4**
DT_{50} [Day]	**7**	**23**
DT_{90} [Day]	**60**	**169**

Conclusion:

In both water/sediment systems S-2354 degraded via the metabolites 2-COOH-S-2200 and 5-COOH-S-2200 which reached maximum levels of 0.5 % AR and 1.7 % AR in the whole system, respectively. Further degradation led to the metabolite MCBX which accounted for up to 18 % AR in the whole system. No other compounds were detected at levels above 2 % of AR in water and / or sediment. Mineralisation over 102 days was relatively small (1.2 – 1.3 % of AR) and not different between the two water/sediment systems. Amounts of bound residues remaining after 102 days were also very similar between the two water/sediment systems being 6.9 – 9.1 % of AR. S-2354 dissipated from the water and reached maximum amounts of 69 % of AR (29 DAT, Calwich Abbey) and 65 % AR (102 DAT, Swiss Lake system) in the sediment phase. Degradation in the whole system was determined using SFO (following guidance document FOCUS, 2006) with first order DT_{50} values of 155 and 600 days and DT_{90} values of 516 and >1000 days.

Comments (RMS):

The study is considered correct.

Reference:	**Calculation of S-2200 sediment water kinetics according to FOCUS (2006) Guidance**
Author(s), year:	Jarvis, T., Mamouni, A., 2012
Report/Doc. number:	ROM-0034
Guideline(s):	FOCUS Degradation Kinetics Report (FOCUS 2006)
GLP:	Not applicable
Deviations:	None
Validity:	Yes

Material and methods:

The active substance S-2200 is a racemic mixture of the two isomers S-2167 (*R*-isomer) and S-2354 (*S*-isomer). Therefore, two separate water sediment studies were conducted using [benzyl-^{14}C] and [phenoxy-^{14}C] labelled S-2167 and [benzyl-^{14}C] labelled S-2354. Full study details are given under water sediment studies above.

The data of the *S*-isomer and of the *R*-isomer were combined to determine the rate of degradation of S-2200 (the racemate) in the overall system or water phase (P-I approaches) and the percentages of metabolites formed. The combined data were obtained by calculating the arithmetic mean of the benzyl and phenoxy labelled S-2167 and by calculating the arithmetic mean of these data and the benzyl labelled S-2354. Then the combined data (expressed as % of AR) were fitted to SFO, FOMC and DFOP kinetics, according to FOCUS (2006) guidance.

The kinetic modelling analysis of the combined residue data was conducted using KinGui Version 2.

Findings:

The combined data of the *S*-isomer and *R*-isomer used for kinetic modelling are presented in Table 97 for Calwich Abbey system and in Table 98 for Swiss lake system. The optimised parameters and associated statistics for FOMC and DFOP kinetics in the water phase and for SFO kinetics for the whole system are shown in Table 99.

Table 97: Distribution of Radioactivity following incubation as S-2200 in Calwich Abbey water sediment system

Time (day)	Water			Sediment			Total		
	S-2200	5-COOH-S-2200	MCBX	S-2200	5-COOH-S-2200	MCBX	S-2200	5-COOH-S-2200	MCBX
0	94.6	ND	ND	2.2	NA	NA	96.8	ND	ND
0	94.5	ND	ND	3.5	NA	NA	97.9	ND	ND
mean	94.5	ND	ND	2.8	NA	NA	97.4	ND	ND
7	51.1	ND	ND	44.6	ND	0.4	95.7	ND	0.4
8	44.5	ND	ND	48.9	0.2	0.7	93.4	0.2	0.7
mean	47.8	ND	ND	46.7	0.1	0.5	94.5	0.1	0.5
14	34.6	0.1	0.1	57.6	0.2	ND	92.2	0.3	0.1
14	34.0	ND	0.2	58.6	0.1	1.0	92.6	0.1	1.2
mean	34.3	0.1	0.1	58.1	0.1	0.5	92.4	0.2	0.7

31	18.2	0.1	0.2	70.0	0.4	1.6	88.2	0.4	1.8
31	17.2	0.1	0.4	69.4	0.4	2.0	86.6	0.5	2.4
mean	17.7	0.1	0.3	69.7	0.4	1.8	87.4	0.5	2.1
62	8.4	ND	1.0	66.5	0.9	7.4	74.9	1.0	8.4
62	8.9	1.1	0.5	65.3	2.4	7.7	74.2	3.5	8.1
mean	8.7	0.6	0.7	65.9	1.7	7.5	74.5	2.2	8.3
102	8.6	1.5	1.3	63.1	0.8	7.8	71.6	2.3	9.0
102	6.9	0.4	1.4	65.4	0.9	7.7	72.3	1.3	9.1
mean	7.7	0.9	1.4	64.3	0.9	7.7	72.0	1.8	9.1

Table 98: Distribution of Radioactivity following incubation as S-2200 in Swiss Lake water sediment system

Time (day)	Water			Sediment			Total		
	S-2200	5-COOH-S-2200	MCBX	S-2200	5-COOH-S-2200	MCBX	S-2200	5-COOH-S-2200	MCBX
0	94.3	ND	ND	3.5	NA	NA	97.7	ND	ND
0	95.2	ND	ND	2.4	NA	NA	97.7	ND	ND
mean	94.7	ND	ND	3.0	NA	NA	97.7	ND	ND
7	59.6	ND	ND	34.1	ND	ND	93.7	ND	ND
8	61.5	ND	ND	33.9	ND	ND	95.5	ND	ND
mean	60.6	ND	ND	34.0	ND	ND	94.6	ND	ND
14	52.9	ND	ND	40.6	ND	0.2	93.4	ND	0.2
14	52.2	ND	ND	40.3	0.1	0.2	92.5	0.1	0.2
mean	52.5	ND	ND	40.4	0.1	0.2	93.0	0.1	0.2
31	40.7	0.1	0.3	47.7	0.1	0.8	88.4	0.2	1.0
31	40.1	1.0	0.2	48.4	0.2	0.6	88.5	1.2	0.7
mean	40.4	0.6	0.2	48.1	0.2	0.7	88.5	0.7	0.8
62	26.3	1.4	0.4	59.5	1.1	1.8	85.7	2.5	2.2
62	26.7	1.6	0.5	61.8	0.6	1.2	88.4	2.3	1.7
mean	26.5	1.5	0.5	60.6	0.8	1.5	87.1	2.4	2.0
102	19.4	1.0	0.1	64.9	0.6	1.4	84.4	1.8	1.5
102	19.9	1.6	0.3	65.0	0.6	1.6	84.8	2.4	1.9
mean	19.6	1.3	0.2	64.9	0.6	1.5	84.6	2.1	1.7

Table 99: Dissipation and degradation rates of S-2200 in water sediment systems

	Parameter	Calwich Abbey	Swiss Lake
Dissipation from water phase	*Model*	*FOMC*	*DFOP*
	χ^2 error (%)	2.83	2.59
	DT_{50} (day)	7.8	19.0
	DT_{90} (day)	64.8	157.7
Degradation in system	*Model*	SFO	SFO
	χ^2 error (%)	2.02	1.57
	DT_{50} (day)*	212 (7.2×10^{-8})	519 (1.7×10^{-5})
	DT_{90} (day)	703	1725

*P value from t-test given in brackets

In the overall system the χ^2 error (%) value for SFO showed a very good fit, and this was also visually acceptable. Hence no further kinetic methods were considered. In the Calwich Abbey system a DT_{50} value of 212 days was calculated and in the Swiss lake system a DT_{50} value of 519 days was calculated. The respective DT_{90} values were 703 and 1725 days. The majority of S-2200 occurs in the sediment and hence for simulation value, the whole system DT_{50} should be used for sediment phase. The default DT_{50} of 1000 days is then used for the water phase.

For the water dissipation phase, SFO kinetics did not give a good fit and the best fit (visually and by the χ^2 error (%) value) was FOMC in Calwich Abbey and DFOP in Swiss lake. In the Calwich Abbey system a DT_{50} value of 7.8 days and a DT_{90} value of 64.8 days was calculated and in the Swiss lake system a DT_{50} value of 19 days and a DT_{90} value of 158 days was calculated.

Conclusion

Two water sediment studies, one with each isomer (S-2167 and S-2354), were conducted in order to address the biological degradation behavior of the active substance S-2200 (a racemic mixture of S-2167 and S-2354) in aquatic systems. The data of the *S*-isomer and of the *R*-isomer were combined to determine the rate of degradation of S-2200 (the racemate) in the overall system or water phase (P-I approaches) and the percentages of metabolites formed. For the water dissipation phase FOMC in Calwich Abbey and DFOP in Swiss lake showed best fit and DT_{50} values of 7.8 and 19 days and DT_{90} values of 64.8 and 158 days were obtained. In the overall system SFO kinetics showed a good fit and DT_{50} values of 212 and 519 days (geometric mean: 332 days) and DT_{90} values of 703 and 1725 days were obtained. The majority of S-2200 occurs in the sediment and hence for simulation values, the **whole system DT_{50} of 332 days (geometric mean)** should be used for the sediment phase. The default **DT_{50} of 1000 days** is then used for the **water** phase. No metabolite exceeds 10% in the overall system but MCBX reaches a maximum of 9.1% after 102 days.

5.1.3 Summary and discussion of degradation

The active substance S-2200 is a racemic mixture of the *R*-isomer (S-2167) and the *S*-isomer (S-2354). In order to address the fate and behaviour of S-2200 in aquatic systems, the hydrolysis, photolysis and biological degradation of both the *R*-isomer and *S*-isomer have been assessed separately. The isomers showed comparable behaviour concerning hydrolytic and photolytic degradation, biological degradation and degradation in water sediment systems. No isomerisation between the S-2200 *R*- and *S*- isomers was observed in any of the aquatic studies.

Aquatic hydrolysis

Two hydrolysis studies in dark sterile buffer solutions at pH 4, 7 and 9 at 50 °C using [benzyl-^{14}C] labelled S-2167 and [benzyl-^{14}C] labelled S-2354 were carried out. Both the S-2200 *R*-isomer and *S*-2200 *S*-isomer were hydrolytically stable at pH 4, 7 and 9 at 50°C. According to OECD 111 the expected DT_{50} at 25°C would be >1 year for each isomer and hence for the racemate, S-2200. No hydrolysis of S-2167 and of S-2354 would be expected under environmental conditions.

Aquatic photolysis

Aquatic photolysis of S-2200 was investigated in sterile buffer solutions at pH 7.0 using [benzyl-^{14}C] and [phenoxy-^{14}C] labelled *R*-isomer (S-2167) and [benzyl-^{14}C] labelled *S*-isomer (S-2354). The test systems were continuously irradiated with a xenon lamp (> 290 nm) for 30 days at 25 °C

to simulate the impact of natural light. Under irradiation in sterile buffer solutions at pH 7.0, both the S-2200 *R*-isomer and S-2200 *S*-isomer degraded rapidly. SFO DT_{50} values of 4.4-4.6 days (equivalent to the same number of days of natural summer sunlight in UK/US) were obtained. Appropriate controls confirmed that there was no degradation in darkness. For both isomers the main photolytic products were S-2200-OR and S-2200-ORC. The mean maximum value for the metabolite levels across both isomers and labelling positions (i.e. 20.7% after 7 days for S-2200-OR and 13.7 % after 14 days for S-2200-ORC) were considered appropriate values for exposure assessments arising from use of S-2200. For the calculation of the overall maximum value the arithmetic mean of the [benzyl-^{14}C] and [phenoxy-^{14}C] labelled *R*-isomer (S-2167) was calculated first; from the maximum mean value of S-2167 and the maximum value of S-2354 the arithmetic mean was calculated which is the overall maximum mean value of the metabolite. A mean SFO DT_{50} value of 4.0-5.1 days was calculated for S-2200-OR, whereas S-2200-ORC appeared stable to photolysis. Two other products were identified for both isomers, namely S-2200-PR and De-Xy-S-2200. The metabolite S-2200-PR reached up to 9.6 % AR after 4 days and was shown to rapidly photodegrade with SFO DT_{50} values of 2.2-2.5 days. The metabolite De-Xy-S-2200 reached up to 7.2 % AR after 30 days and no degradation rate could be calculated. Extensive breakdown of the molecule structure was observed after 30 days incubation as evidenced by the large number of minor unknowns which individually reached up to max. 8.1 % AR after 21 days. The quantum yield for S-2200 *R*-isomer and S-2200 *S*-isomer were determined to be 0.283 and 0.269, respectively.

Biological degradation

Results of a **readily biodegradability study** indicate that S-2200 is not readily biodegradable.

Two water sediment studies, one with each isomer (S-2167 and S-2354), were conducted in order to address the biological degradation behavior of the active substance S-2200 (a racemic mixture of S-2167 and S-2354). The dark aerobic **water/sediment studies** were conducted with two contrasting (pH, texture) natural systems, Calwich Abbey and Swiss Lake, using [benzyl-^{14}C] and [phenoxy-^{14}C] labelled S-2167 and [benzyl-^{14}C] labelled S-2354. The Calwich Abbey test system represents a silt loam sediment with an organic carbon content of 5.0 %, a microbial biomass of 1561 µg C g^{-1} and a pH of 7.5 ($CaCl_2$). The Swiss Lake test system is characterized by a loamy sand sediment with a pH of 5.1 ($CaCl_2$), with an organic carbon content of 0.9 % and a lower microbial biomass (356 µg C g^{-1}). Both systems stayed aerobically throughout the test period.

Mineralisation of S-2167 using [benzyl-^{14}C] label and [phenoxy-^{14}C] label accounted by study termination for maximum 1.4 and 2.4 % of AR in the Calwich Abbey system and 3.7 and 2.3 % of AR in the Swiss lake system, respectively. Mineralisation of [benzyl-^{14}C] labelled S-2354 accounted for 1.2 and 1.3 % AR by study termination in the Calwich Abbey system and in the Swiss lake system, respectively. Thus, mineralisation was not very different between the two radiolabelled forms, between the two isomers and between the two water/sediment systems.

Formation of NER increased up to maximum 7.9 % of AR with [benzyl-^{14}C] labelled S-2167 and to 7.8 % of AR with [phenoxy-^{14}C] labelled S-2167 in the Calwich Abbey system, and to 5.8 % of AR with [benzyl-^{14}C] labelled S-2167 and to 5.1 % of AR with [phenoxy-^{14}C] labelled S-2167 in the Swiss lake system. With [benzyl-14C] labelled S-2354 the formation of NER increased up to 9.1 % AR in the Calwich Abbey system and up to 6.9 % AR in the Swiss lake system. Thus, amounts of bound residues remaining after 100 days were also very similar between the two radiolabelled forms, between the two isomers and between the two water/sediment systems.

Both isomers dissipated relatively fast from the water into the sediment. Maximum occurrence of [benzyl-^{14}C] labelled S-2167 in sediment was 73.2 % AR (DAT 62) and 62.1 % AR (DAT 101)

in Calwich Abbey and Swiss Lake respectively. For [phenoxy-^{14}C] labelled S-2167 the maximum occurrence in sediment was 72.9 % of AR (DAT 31) and 67.3 % of AR (DAT 101) in Calwich Abbey and Swiss Lake respectively. Maximum occurrence of [benzyl-^{14}C] labelled S-2354 in sediment was 69 % AR (DAT 29) and 65 % AR (DAT 102) in Calwich Abbey and Swiss Lake respectively. No isomerisation of S-2167 and of S-2354 occurred.

Degradation in the whole system was determined using SFO with first order DT_{50} values of 342 and 344 days for the [benzyl-^{14}C] labelled S-2167 and with first order DT_{50} values of 284 and 654 days for the [phenoxy-^{14}C] labelled S-2167 in Calwich Abbey and Swiss Lake, respectively. The respective DT_{90} values were in the range of 942 - >1000 days. For [benzyl-^{14}C] labelled S-2354 first order DT_{50} values of 155 and 600 days and DT_{90} values of 516 and >1000 days were determined in Calwich Abbey and Swiss Lake, respectively. The DT_{50} and DT_{90} values of dissipation and degradation of both isomers are summarized in table 100. The degradation behavior of [benzyl-^{14}C] labelled S-2167 was very similar in the two systems, whereas the degradation rate of [phenoxy-^{14}C] labelled S-2167 showed a higher variety in the two systems. Comparing the two labels it could be shown that [phenoxy-^{14}C] labelled S-2167 degraded faster in Calwich Abbey system and slower in Swiss Lake system. Comparing the two isomers it can be seen that [benzyl-^{14}C] labelled S-2354 degraded faster in Calwich Abbey system but as slow as [phenoxy-^{14}C] labelled S-2167 in Swiss Lake system. Considering the fact that about 80 % of the substance was not degraded by study termination and that the calculated DT_{50} values are extrapolated past the incubation time it was concluded that the degradation behavior of the two isomers in water sediment systems was comparable.

To determine the rate of degradation of S-2200 (the racemate) in the overall system or water phase (P-I approaches) the data of the *S*-isomer and of the *R*-isomer were combined. For the water dissipation phase FOMC in Calwich Abbey and DFOP in Swiss lake showed best fit and DT_{50} values of 7.8 and 19 days and DT_{90} values of 64.8 and 158 days were obtained. In the overall system SFO kinetics showed a good fit and DT_{50} values of 212 and 519 days (geometric mean: 332 days) and DT_{90} values of 703 and 1725 days were obtained for Calwich Abbey and Swiss Lake system, respectively. The DT_{50} and DT_{90} values of dissipation and degradation of S-2200 are summarized in table 101. The majority of S-2200 occurs in the sediment and hence for simulation values, the whole system DT_{50} of 332 days (geometric mean) should be used for the sediment phase. The default DT_{50} of 1000 days is then used for the water phase.

In both water/sediment systems and for both labels S-2167 as well as S-2354 degraded via the metabolites 2-COOH-S-2200 and 5-COOH-S-2200, which reached maximum levels of 1.9 % AR and 6.6 % AR in the whole system, respectively. Further degradation led to the metabolite MCBX which accounted for < 1 % AR for S-2167. For S-2354 the metabolite MCBX exceeded 10 % AR in Calwich Abbey system and accounted for up to 15.4 % AR in sediment and up to 18 % AR in the whole system. Since the active substance S-2200 is a racemic mixture of both isomers the data of the *S*-isomer and of the *R*-isomer were combined to determine the percentages of metabolites formed. Thus, no metabolite exceeds 10% in the overall system but MCBX reaches a maximum of 9.1% after 102 days.

Table 100: Summary on DT_{50} and DT_{90} [days] for the dissipation and degradation of benzyl and phenoxy labelled S-2167 and benzyl labelled S-2354 in aerobic water/sediment studies.

		S-2167, [Benzyl-^{14}C] labelled		**S-2167, [Phenoxy-^{14}C] labelled**		**S-2354, [Benzyl-^{14}C] labelled**	
	Parameter	**Calwich Abbey**	**Swiss Lake**	**Calwich Abbey**	**Swiss Lake**	**Calwich Abbey**	**Swiss Lake**
Dissipation from water phase	*Model*	*FOMC*	*FOMC*	*FOMC*	*FOMC*	*FOMC*	*DFOP*
	χ^2 error (%)	1.87	3.71	5.66	2.73	3.5	3.4
	DT_{50} (day)	9	15	7	17	7	23
	DT_{90} (day)	83	333	56	416	60	169
Degradation in whole system	*Model*	*SFO*	*SFO*	*SFO*	*SFO*	*SFO*	*SFO*
	χ^2 error (%)	1.14	2.2	3.15	1.47	2.3	1.6
	DT_{50} (day)*	342 (1×10^{-6})	344 (1.3×10^{-5})	284 (3×10^{-4})	654 (1.2×10^{-4})	155 (7.9×10^{-8})	600 (2.3×10^{-4})
	DT_{90} (day)	>1000	>1000	942	>1000	516	>1000

*P value from the t-test is given in brackets.

Table 101: Summary on DT_{50} and DT_{90} [days] for the dissipation and degradation of S-2200 in aerobic water sediment systems

		S-2200	
	Parameter	**Calwich Abbey**	**Swiss Lake**
Dissipation from water phase	*Model*	*FOMC*	*DFOP*
	χ^2 error (%)	2.83	2.59
	DT_{50} (day)	7.8	19.0
	DT_{90} (day)	64.8	157.7
Degradation in system	*Model*	*SFO*	*SFO*
	χ^2 error (%)	2.02	1.57
	DT_{50} (day)*	212 (7.2×10^{-8})	519 (1.7×10^{-5})
	DT_{90} (day)	703	1725

*P value from t-test given in brackets

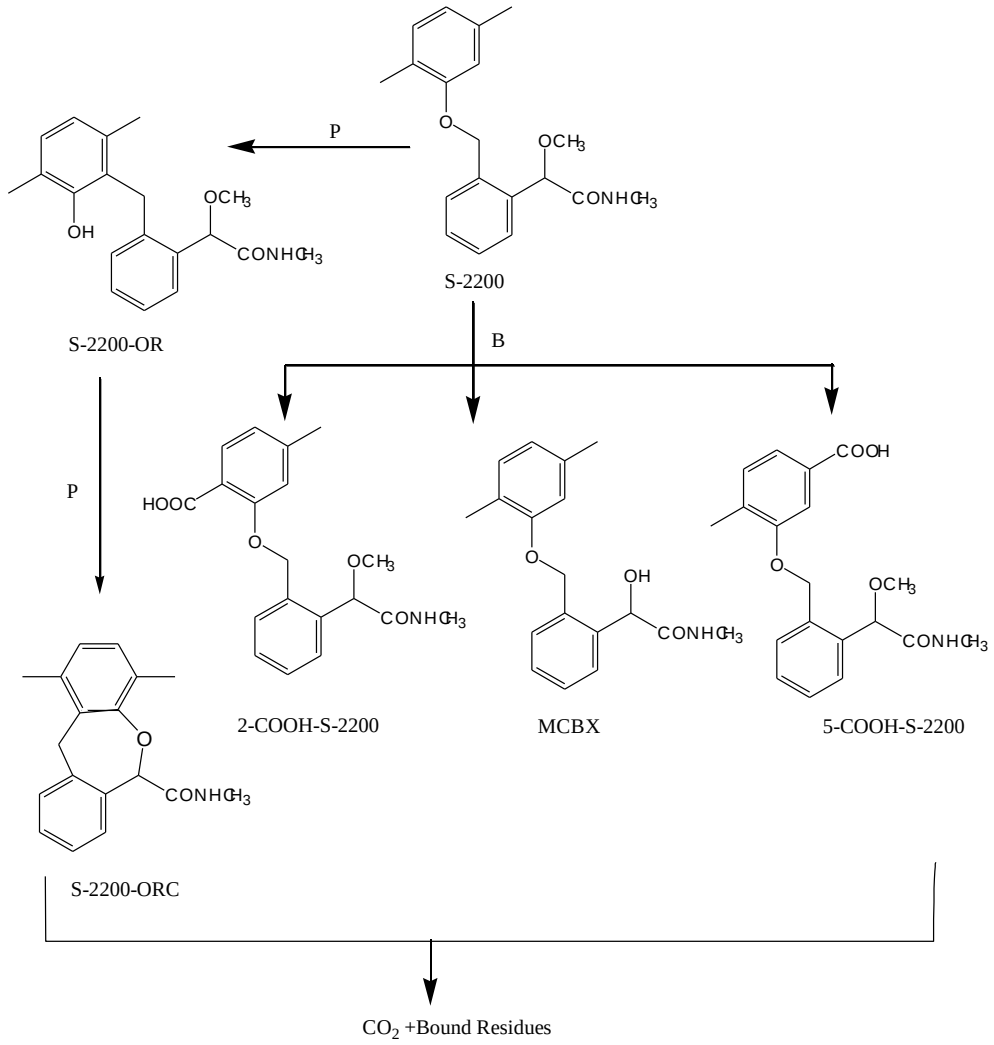


Figure 5: Proposed aquatic degradation routes of S-2200 (B=biotic and P=photolytic)

5.2 Environmental distribution

The active substance S-2200 (mandestrobin) is a racemic mixture (1:1) of the two isomers S-2167 (S-2200 *R*-isomer) and S-2354 (S-2200 *S*-isomer). In order to determine the route and rate of degradation of the active substance mandestrobin (S-2200) in soil separate studies with the two isomers were conducted. No isomerisation occurred during any of the studies.

Route of degradation in soil

The **route** of degradation under **aerobic conditions** was assessed for both isomers (S-2167 and S-2354) separately on six EU soils using [benzyl-^{14}C] and [phenoxy-^{14}C] labelled S-2167 and [benzyl-^{14}C] labelled S-2354. The route of degradation was qualitatively and quantitatively the same for both isomers.

Under standard aerobic conditions, S-2167 and S-2354 degraded mainly to CO_2, bound residues and up to five metabolites. Levels of CO_2 after 120 days incubation were in the range of 4.2 – 34.4 % of AR for S-2167 and of 4.4 –27.1 % of AR for S-2354. Levels of bound residues increased steadily during the study period to reach at the end of the study values between 6.7 % and 33.2 % of AR for S-2167 and of 6.4 % and 29.7 % of AR for S-2354. The metabolite 5-

COOH-S-2200 was detected as a major metabolite reaching up to a mean maximum of 19.7 % AR for S-2167 and of 16.1 % AR for S-2354 after 59 and 60 d. The metabolite 2-COOH-S-2200 was present at ≥ 5% and ≤ 10 % with a mean maximum of 8.7 % of AR after 60 d for S-2167 and of 4.8 % AR after 120 d for S-2354. The metabolite DX-CA-S-2200/De-Xy-S-2200 was detected at a maximum of 4.1 % after 120 d and the metabolites MCBX and De-Xy-S-2200 did not exceed 1% in any soil at any timepoint. All other metabolites individually accounted for less than 5 %.

Based on the combined results for the racemate, the metabolite **5-COOH-S-2200** reached a maximum of **18.0 %** and the metabolite **2-COOH-S-2200** reached a maximum of **6.7 %**. A proposed degradation pathway for S-2200 is shown in Figure 6.

No **anaerobic degradation** study was presented as the substance is to be applied to winter oil seed rape during later spring (in North and South Europe). Therefore it is unlikely to be present in soil during waterlogged (anaerobic) conditions in winter and hence an anaerobic soil degradation study was not considered relevant.

The **photolytic degradation** of S-2167 (S-2200 *R*-isomer) and S-2354 (S-2200 *S*-isomer) was assessed in one soil. The route of photolytic degradation was very similar for both isomers, both isomers degraded when exposed to light on soil surface. The route of degradation was similar in samples exposed to light and dark controls. No major metabolite (> 10 % of AR) was detected in the irradiated samples or in dark controls.

The route and rate of degradation under aerobic conditions of the soil **metabolites 2-COOH-S-2200 and 5-COOH-S-2200** were assessed in separate studies on three European soils. The route of degradation was very similar for the two metabolites.

Both metabolites degraded mainly to CO_2 and bound residues. Levels of CO_2 after 120 days incubation were in the range of 43 to 54 % of AR for 2-COOH-S-2200 and of 38 to 52 % of AR for 5-COOH-S-2200. Levels of bound residues were between 37 % and 48 % of AR at the completion of the 120 days incubation. The metabolite DX-CA-S-2200 was identified but reached < 2 % of AR in any soil. Up to four unknown peaks represented a total of 11.7 % of AR, but no individual metabolites were present at ≥ 6 % AR.

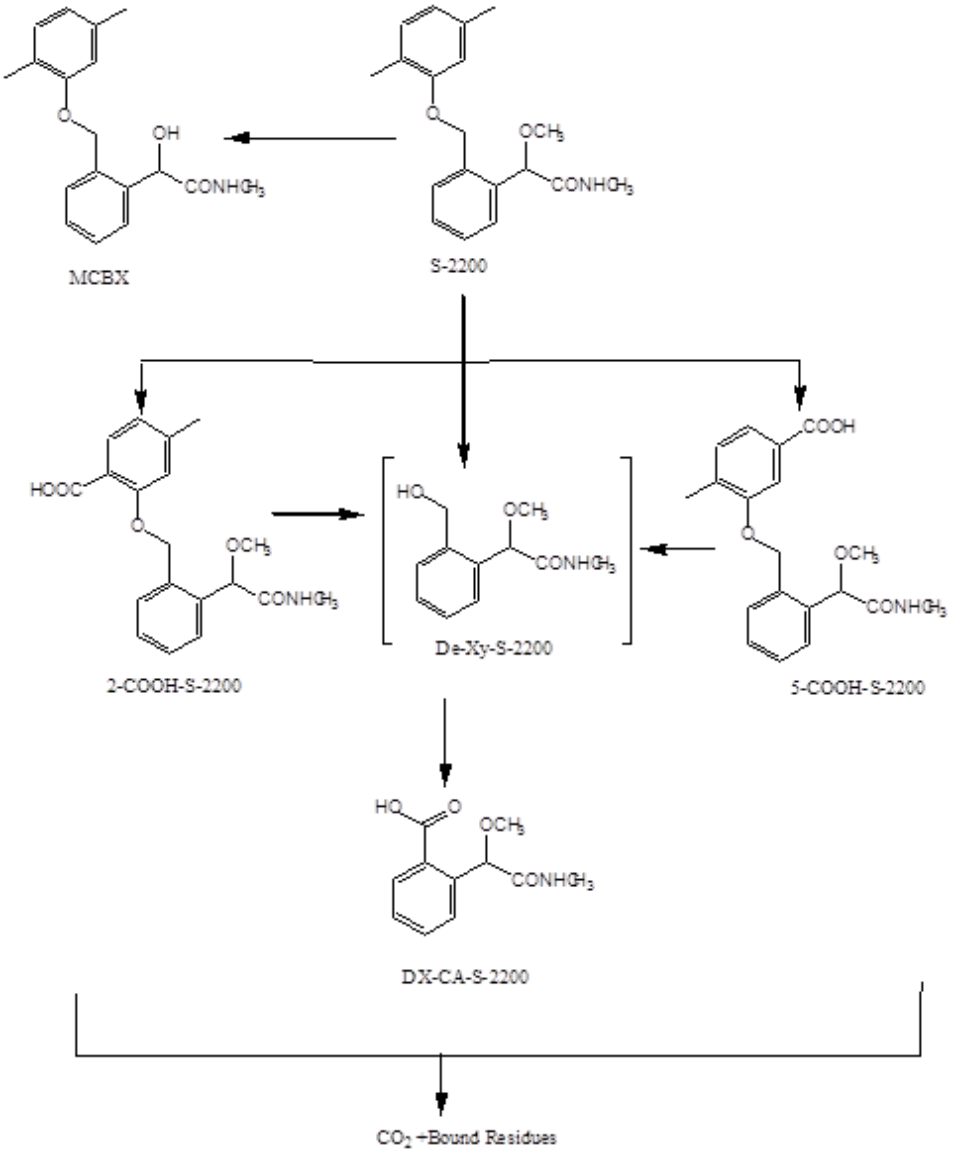


Figure 6: Proposed metabolic pathway for Mandestrobin (S-2200) in soil

Rate of degradation in laboratory studies

The laboratory soil **degradation rate** of the two isomers S-2167 (S-2200 *R*-isomer) and S-2354 (S-2200 *S*-isomer) of S-2200 was investigated separately in six EU soils. The *R*-isomer **S-2167** degraded with SFO DT_{50} values in the range of 40 to 227 days and DT_{90} values in the range of 132 to 754 days. The SFO DT_{50} and DT_{90} values of the *S*-isomer **S-2354** were in the range of 60 to 323 days and of 200 to > 1000 days, respectively. The results indicated that S-2167 degraded faster than S-2354. Since S-2167 represents the active part of S-2200 it is considered acceptable to combine the residues of the two isomers for determination of the degradation rate for S-2200.

For the determination of the degradation rate (for use as simulation input value) of **S-2200** the residue data of the two isomers S-2167 and S-2354 were combined. In four soils degradation was clearly seen to follow SFO kinetics and the DT_{50} values calculated for S-2200 were between 50 and 76 days. For two additional soils DFOP kinetics provided better fits for visual and statistical assessment. The FOCUS (2006) document recommends in the first instance to use the slow phase

from the DFOP kinetics as input value for modelling (first order value). The respective DT_{50} values were 184 and 415 days. During the peer review information on the potential correlation of soil DT_{50} values for mandestrobin with soil properties, in particular with soil pH was requested. The statistical assessment of the DT50 values (modelling endpoints) and soil properties showed that there are some indications that the degradation of mandestrobin in soil is pH-dependent. Therefore, a DT50 value of **276.44 d for acidic soils** (pH≤5.9, n=2) and a DT50 value of **59.52 d for alkaline soils** (pH≥7.2, n=4) were calculated by the RMS.

In order to determine the degradation rate and formation fractions (for use as simulation input values) of the metabolites **2-COOH-S-2200 and 5-COOH-S-22000** simultaneous fitting for parent parameters and DT_{50} and formation fractions of the metabolites were used based on the previously chosen kinetic order for the parent.

Additionally, the degradation behaviour of the metabolite **2-COOH-S-2200** and of the metabolite **5-COOH-S-2200** was determined in three European soils. The first order DT_{50} values calculated for 2-COOH-S-2200 were in the range of 18.1 to 25.9 days and the first order DT_{50} values calculated for 5-COOH-S-2200 were the range of 21.9 to 41.0 days. For the determination of the geometric mean DT_{50} values for the metabolites 2-COOH-S-2200 and 5-COOH-S-2200 the studies with the parent as well as the studies with the metabolites applied as parent were considered. The **geometric mean DT_{50} values** for **2-COOH-S-2200** and **5-COOH-S-2200** are determined to be **28.68 days** and **36.91 days**, respectively.

Photodegradation of S-2167 and S-2354 on soil surface exposed to light was investigated in one soil. The rate of degradation in soils that were exposed to light was accelerated compared to dark controls. The DT50 values for S-2167 exposed to light were 49 and 56 days for [benzyl-^{14}C] and [phenoxy-^{14}C] label and the DT_{50} values for S-2167 in the dark controls were determined to be 62 and 85 days for [benzyl-^{14}C] and [phenoxy-^{14}C] label. The DT_{50} value for S-2354 exposed to light was 64 days and the DT50 value for S-2354 in the dark controls was determined to be 83 days.

Table 102: Summary of DT50 values of mandestrobin and its metabolites 2-COOH-S-2200 and 5-COOH-S-2200

Soil type	**OC [%]**	**pH ($CaCl_2$)**	**Temperature [°C] / soil moisture [MWHC]**	**DT_{50} /DT_{90} [d]**	**DT_{50} [d]** 20°C pF2/10kPa**	**X^2 [%]**	**ff**	**Method of calculation**	**remark**
Mandestrobin									
Speyer 5M	1.3	7.2	20 °C/pF2	76.1/252.8	76.1	4.3/2.4*		SFO	Parent fitted with metabolites
Speyer 2.2	2.1	5.5	20 °C/pF2	335/-	415.1	3.7/2.4*		DFOP	
SK920191	3.8	7.6	20 °C/pF2	53.1/176.4	53.1	11.1/6.7*		SFO	
Chelmorton (SK104691)	3.4	5.9	20 °C/pF2	102.8/530.3	184.1	3.8/2.4*		DFOP	
Aschard	1.3	7.4	20 °C/pF2	62.5/207.5	62.5	4.95/3.3*		SFO	
Monteil	1.4	7.7	20 °C/pF2	49.7/165.2	49.7	5.1/3.2*		SFO	
Geometric mean, acidic soils (pH ≤5.9)					**276.44**				n=2
Geometric mean, alkaline soils (pH ≥7.2)					**59.52**				n=4
2-COOH-S-2200									
Speyer 5M	1.3	7.2	20 °C/pF2	20.6/68.3	20.6	4.6	0.2848	SFO	Fitted with parent
Speyer 5M	1.2	7.2	20 °C/pF2	25.9/86.1	25.9	4.01		SFO	Metabolite as parent
Speyer 2.2	2.1	5.5	20 °C/pF2	>>1000/ >>1000	>>>1000***	1.8	-	SFO	Fitted with parent
SK920191	3.8	7.6	20 °C/pF2	15.3/50.9	15.3	9.9	0.3035	SFO	Fitted with parent
SK920191	4.1	7.5	20 °C/pF2	20.4/67.8	20.4	2.03		SFO	Metabolite as parent
Chelmorton (SK104691)	3.4	5.9	20 °C/pF2	177.3/588.8	177.3	11.2	0.1306	SFO	Fitted with parent
SK104691	3.2	5.6	20 °C/pF2	18.1/60.0	18.1	3.36		SFO	Metabolite as parent
Aschard	1.3	7.4	20 °C/pF2	29.5/97.9	29.5	4.3	0.3076	SFO	Fitted with parent
Monteil	1.4	7.7	20 °C/pF2	29.0/96.5	29.0	3.0	0.1665	SFO	Fitted with parent
Geometric mean					**28.68**				n=8
Arithmetic mean							**0.2386**		n=5
5-COOH-S-2200									
Speyer 5M	1.3	7.2	20 °C/pF2	33.6/111.5	33.6	8.6	0.5349	SFO	Fitted with parent
Speyer 5M	1.2	7.2	20 °C/pF2	41.0/136.1	41.0	2.37		SFO	Metabolite as parent
Speyer 2.2	2.1	5.5	20 °C/pF2	52.3/173.9	52.3	3.0	0.4092	SFO	Fitted with parent
SK920191	3.8	7.6	20 °C/pF2	20.4/67.9	20.4	6.1	0.6965	SFO	Fitted with parent
SK920191	4.1	7.5	20 °C/pF2	30.3/100.5	30.3	7.95		SFO	Metabolite as parent

Soil type	**OC [%]**	**pH ($CaCl_2$)**	**Temperature [°C] / soil moisture [MWHC]**	**DT_{50} /DT_{90} [d]**	**DT_{50} [d]** 20°C pF2/10kPa**	**X^2 [%]**	**ff**	**Method of calculation**	**remark**
Chelmorton (SK104691)	3.4	5.9	20 °C/pF2	136.8/454.4	136.8	2.1	0.2052	SFO	Fitted with parent
SK104691	3.2	5.6	20 °C/pF2	21.9/72.7	21.9	4.5		SFO	Metabolite as parent
Aschard	1.3	7.4	20 °C/pF2	38.3/127.1	38.3	1.4	0.6924	SFO	Fitted with parent
Monteil	1.4	7.7	20 °C/pF2	24.9/82.7	24.9	2.9	0.4747	SFO	Fitted with parent
Geometric mean					**36.91**				n=9
Arithmetic mean							**0.502**		n=6

*all data/S-2200
**slow rate k value of DFOP kinetics
***Value not used for determination of geometric mean due to low occurrence of the metabolite in this soil (≤3 % AR)

Field dissipation studies

A field study was undertaken at four sites (France, Spain, Austria, Germany). The non-normalised data were best fitted with DFOP kinetics. For the kinetic evaluation of S-2200 the sum of S-2167 and S-2354 residues was used.The DT_{50} values obtained were in the range of 1.8 days to 9.4 days and the DT_{90} values were in the range of 45.5 days to 281.0 days. From the residues detected in soil samples and from the calculated dissipation rates it can be concluded that the **DT_{90} in field is below 365 days** and that no further studies are required.

The determined residue levels (0-30 cm soil horizon) of S-2167, S-2354 and of the metabolites De-Xy-S-2200, 2-COOH-S-2200 and 5-COOH-S-2200 indicate that movement to lower soil layers is not expected. The metabolites De-Xy-S-2200, 2-COOH-S-2200 and 5-COOH-S-2200 reached up to 0.011 mg kg^{-1} in 0-10 cm horizon, no clear trends of formation and decline of the metabolites could be observed.

Table 103: Dissipation DT_{50} and DT_{90} values for S-2167, S-2354 and S-2200

Field site	DT_{50} [days]	DT_{90} [days]	Error level χ^2 [%]	Kinetic
S-2167				
French site (Site 8202031/1)	2.61	46.46	11.27	DFOP
Spanish site (Site 8202031/2)	2.69	49.39	11.2	DFOP
Austrian site (Site 8202031/3)	9.35	45.46	8.9	DFOP
German site (Site 8202031/4)	4.01	174.97	30.20	DFOP
S-2354				
French site (Site 8202031/1)	1.84	50.57	11.69	DFOP
Spanish site (Site 8202031/2)	2.92	66.05	8.57	DFOP
Austrian site (Site 8202031/3)	8.6	107.51	7.66	DFOP
German site (Site 8202031/4)	4.74	280.99	28.76	DFOP
S-2200				
French site (Site 8202031/1)	2.29	47.92	11.14	DFOP
Spanish site (Site 8202031/2)	2.82	55.09	8.73	DFOP
Austrian site (Site 8202031/3)	8.28	81.63	5.72	DFOP
German site (Site 8202031/4)	4.53	225.93	29.11	DFOP

5.2.1 Adsorption/Desorption

The **sorption of S-2200** was studied in four European soils and one Japanese soil (pH range 4.0 to 7.4, C_{org} range 1.3 – 5.0 %, clay 9 – 28 %). Adsorption **K_{Foc} values** were between 287 and 797 ml g^{-1} (mean **449** ml g^{-1}, mean **1/n = 0.92**) and desorption $K_{Foc-des}$ values were between 340 and 1003 ml g^{-1} (mean 556 ml/g, mean 1/n= 0.91) indicating that S-2200 is low to medium mobile. There was no evidence of any pH dependence.

Table 104: Adsorption/desorption characteristics of [14C]S-2200 on five soils

Soil	Organic carbon [%]	pH ($CaCl_2$)	Adsorption			Desorption		
			K_F^{ads}	K_{Foc}	1/n	K_F^{des}	$K_{Foc-des}$	1/n
SK961089	5.0	7.4	15	296	0.9163	17	340	0.8949
SK104691	2.5	6.1	7	287	0.9315	9	378	0.9398

Soil	Organic carbon [%]	pH ($CaCl_2$)	Adsorption			Desorption		
			K_F^{ads}	K_{Foc}	1/n	K_F^{des}	$K_{Foc-des}$	1/n
SK179618	3.9	5.0	18	466	0.9615	22	562	0.9584
SK566696	1.3	4.0	10	797	0.8981	13	1003	0.8856
Saitama	3.1	5.6	12	397	0.8888	15	497	0.8910
Arithm. mean (n=5)			12	**449**	**0.9192**	15	**556**	**0.9139**

Soil batch equilibrium experiments were also conducted on the soil metabolites 2-COOH-S-2200 and 5-COOH-S-2200 of mandestrobin using six European soils (pH range 4.0 – 7.4, C_{org} range 1.3 – 5.0 %). The results gave a clear evidence of pH dependence of sorption with the acidic soil showing greater sorption than the neutral/alkaline soils.

For the metabolite **2-COOH-S-2200** the K_{FOC} value was 226 L kg-1 in the acidic soil and the K_{FOC} values for moderately acid/neutral/alkaline soils were in a range of 6 - 24 L kg^{-1} with an arithmetic mean of 15 L kg^{-1} and a mean 1/n of 0.88. Based on these values, 2-COOH-S-2200 is classified as medium mobile in acidic soils and as very high mobile in neutral/alkaline soils. For the metabolite **5-COOH-S-2200** the K_{FOC} value was 684 L kg-1 in the acidic soil and the K_{FOC} values for moderately acid/neutral/alkaline soils were in a range of 29 – 74 L kg^{-1} with an arithmetic mean of 55 L kg^{-1} and mean 1/n of 0.89. Based on these values, 5-COOH-S-2200 is classified as low mobile in acidic soils and high to very high mobile in neutral/alkaline soils.

Table 105: Adsorption/desorption characteristics of [^{14}C]2-COOH-S-2200 on six soils

Soil	Organic carbon [%]	pH ($CaCl_2$)	Adsorption			Desorption		
			K_F^{ads}	K_{Foc}	1/n	K_F^{des}	$K_{Foc-des}$	1/n
Acidic soils								
SK566696	1.3	4.0	2.94	226	0.9225	3.57	274	0.9217
Moderately acid/neutral/alkaline soils								
SK104691	2.5	6.0	0.27	11	0.9158	0.32	13	0.9043
Land Look 250	3.3	6.1	0.46	14	0.8556	0.52	16	0.8536
Land Look 301	3.0	6.5	0.58	19	0.8603	0.71	24	0.8497
Land Look 308	1.4	6.4	0.34	24	0.8861	0.40	29	0.9004
SK961089	5.0	7.4	0.28	6	0.9031	0.33	7	0.9062
Arithm. mean (n=5)			0.39	**15**	0.8842			

Table 106: Adsorption/desorption characteristics of [^{14}C]5-COOH-S-2200on six soils

Soil	Organic carbon [%]	pH ($CaCl_2$)	Adsorption			Desorption		
			K_F^{ads}	K_{Foc}	1/n	K_F^{des}	$K_{Foc-des}$	1/n
Acidic soils								
SK566696	1.3	4.0	8.9	684	0.9259	10.98	844	0.9026
Moderately acid/neutral/alkaline soils								
SK104691	2.5	6.0	1.35	54	0.8822	1.64	66	0.8576
Land Look 250	3.3	6.1	1.8	55	0.8861	2.12	64	0.8899
Land Look 301	3.0	6.5	1.91	64	0.9081	2.6	87	0.9253
Land Look 308	1.4	6.4	1.04	74	0.8955	1.2	85	0.8950
SK961089	5.0	7.4	1.44	29	0.8740	1.59	32	0.8612
Arithm. mean (n=5)			1.51	**55**	0.8892			

5.2.2 Volatilisation

S-2200 has a low vapour pressure of 3.36 x 10^{-8} Pa at 20 °C and is predicted to degrade rapidly in air through reaction with hydroxyl radicals (DT_{50} 1.332 hrs assuming 1 .5 x 10^{6} hydroxyl radicals cm^{-3}). Therefore it is considered that there is no risk of exposure to air and no further data are required.

5.2.3 Distribution modelling

No information available.

5.3 Aquatic Bioaccumulation

Table 107: Summary of relevant information on aquatic bioaccumulation

Method	Results	Remarks	Reference
Partition coefficient n-octanol/water (OPPTS 830.7550, OECD 107, EC Method A8, JMAFF 8147 (shake flask))	log P_{ow} = 3.51 at 25 ± 1 ºC	Test substance: S-2200 PAI, Lot: 081103G Purity: 100%	Van Meter, D.S. & Lentz, N.R., 2010d
	log P_{ow} = 3.44 at 25 ± 1 ºC	Test substance: S-2200 *R*-isomer, Lot: 060020652 Purity: 100%	Van Meter, D.S. & Lentz, N.R., 2010e

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

No estimations are available.

5.3.1.2 Measured bioaccumulation data

Reference:	**Flow-Through Bioconcentration and Metabolism Study of [^{14}C]S-2200 with Bluegill Sunfish (*Lepomis macrochirus*)**
Author(s), year:	Lentz, N. R., 2010
Report/Doc. number:	Report No. ROM-0016, Study No. 13048.6627
Guideline(s):	OECD Guideline 305, US EPA FIFRA 165-4, US EPA OPPTS 850.1730, JMAFF 12-Nosan 8147 2-9-17
GLP:	Yes
Deviations:	None relevant
Validity:	Acceptable

Material and methods:

Test substance: Radiolabeled substance: [Benzyl-^{14}C]S-2200, purity: 98.9 %, batch: CFQ40467
Non-radiolabeled substance: S-2200 PAI, purity: 100 %, batch: 081103G

Reference substances: S-2200, purity: 100 %, batch:081103G
S-2167 (S-2200 *S*-isomer), purity: 100 %, batch: 060020652
S-2354 (S-2200 *R*-isomer), purity: 99.7 %, batch: 060020653
2-COOH-S-2200, purity: 99.8 %, batch: 252-001-60-2
5-COOH-S-2200, purity 99.7 %, batch: 251-011-55-2
2-CH_2OH-S-2200, purity: 98.5 %, batch: CTS08029
5-CH_2OH-S-2200, purity: 99.9 %, batch: CTS08030
4-OH-S-2200, purity: 99.9 %, batch: CTS08026
De-Xy-S-2200, purity: 99.9 %, batch: CTS08001
MCBX, purity: 96.9 %, batch: CTS08015
(*R*)-MCBX, purity: 99.8 %, batch: FUJINAMI
(*S*)-MCBX, purity: 99.4 %, batch: TM633
2,5-dimethylphenol (CAS No.: 95-87-4), purity: 99.8 %, batch: 01197MJ

Test species:	Bluegill Sunfish (*Lepomis macrochirus*)
Number of organisms:	155 fish per test concentration and 154 fish per solvent control, total biomass per aquarium was 146 g (0.24 g/L of the 24-hour flow-through volume of the aquaria. For the depuration phase the remaining fish (59 fish for the solvent control and the 1.0 µg/L treatment, and 58 fish for the 10 µg/L treatment) were placed in clean water for 7 days. 1 replicate per treatment level and solvent control
Weight, length:	Weight: 1.0 g (0.80 – 1.2 g) n = 465 Length: 37 - 45 mm, n = 30
Type of test, duration:	Flow-through test, 28 d exposure period and 7 d depuration period
Applied concentrations:	
Nominal:	0 (solvent control), 1.0 and 10 µg ai/L
Solvent:	Acetone (CAS No. 67-64-1)
Test conditions:	
Water quality:	Well water, total hardness: 60 - 72 mg/L as $CaCO_3$, total alkalinity: 20 – 23 mg/L as $CaCO_3$
Temperature:	22 - 23 °C
pH:	6.1 – 7.2 (exposure phase), 7.0 – 7.3 (depuration phase)
O_2 content:	Exposure phase: 6.1 – 8.6 mg O_2/L (> 60 % saturation) Depuration phase: 7.1 – 8.6 mg O_2/L (> 60 % saturation)
Light regime:	16 hours light / 8 hours darkness
Feeding:	Pelleted food: 1 % of biomass daily
Test parameters:	Residues in water: For chemical analysis (LSC, HPLC/RAM) of S-2200 in test solutions samples were taken at -2 and -1 d (pre-exposure phase), 0, 1, 3, 7, 14, 21 and 28 d (exposure phase) and 1, 3, 7 and 14 d (depuration phase). Residues in fish: Samples were taken at day 0, 1, 3, 7, 14, 21, 24 and 28 (exposure phase) and 1, 3 and 7 (depuration phase). Six fish for tissue analysis were removed from each test concentration and control at each sampling time. Concentration of [^{14}C] S-2200 equivalents in fish tissues were determined by LSC-method. Additionally a lipid analysis (by chloroform/methanol extraction) was carried out on fish sampled at day 1, 3, 7, 14, 21, 24 and 28 (exposure phase) and at day 1, 3 and 7 (depuration phase). Daily observations were made of the appearance and behaviour of the fish. Other parameters like temperature, pH and dissolved oxygen concentrations were measured daily in each vessel.
Calculations/statistics:	BCF was calculated as ratio of [^{14}C] S-2200 equivalents concentration in water and [^{14}C] S-2200 equivalents concentration in fish tissues and as ratio of K_d (depuration constant) and K_u (uptake constant), rate constant K was determined by SigmaPlot™.
Findings:	
Analytical data – water:	Throughout the exposure phase, the radioactivity present in the exposure tanks was confirmed to be more than 95% of [Benzyl-^{14}C]S-2200, and the concentrations of measured S-2200 were 0.989 – 1.061 and 10.053 – 10.965 µg/L for low and high concentrations, respectively. Overall, measured exposure concentrations in the nominal 1.0 and 10 µg/L treatments were between 96.9% and 103.3% of nominal.

Lipid content: During the exposure phase, the percent lipid content based on wet weight for edible, non-edible and whole fish tissue in low and high concentrations ranged from 1.14 – 2.07, 3.43 – 5.51 and 2.45 – 3.63%. During the depuration phase, the percent lipid content based on wet weight for edible, non-edible and whole fish tissue in low and high concentrations ranged from 1.78 – 2.16, 4.64 – 6.23 and 3.16 – 3.94%. Based on these results the lipid content of the whole fish remained relatively consistent over the course of the study and between exposure groups.

Analytical data – fish tissues (LSC): See Table

BCF: See Table

Table 108: Total radioactive residues (TRR) measured in fish tissue (*Lepomis macrochirus*)

Day	Tissue concentration [µg/g]					
	Edible		Non-edible		Whole fish	
	1.0 µg/L	10 µg/L	1.0 µg/L	10 µg/L	1.0 µg/L	10 µg/L
Exposure phase						
1	0.019	0.227	0.138	1.399	0.078	0.835
3	0.023	0.254	0.214	1.592	0.118	0.927
7	0.026	0.226	0.196	1.757	0.112	1.017
14	0.029	0.267	0.220	2.898	0.125	1.571
21	0.038 [a]	0.291 [a]	0.410 [b]	2.416 [b]	0.222 [c]	1.367 [c]
24	0.030 [a]	0.203 [a]	0.350 [b]	2.678 [b]	0.174 [c]	1.458 [c]
28	0.034 [a]	0.258 [a]	0.300 [b]	3.005 [b]	0.163 [c]	1.651 [c]
Depuration phase						
1	0.011	0.056	0.163	1.413	0.086	0.711
3	0.002	0.031	0.065	0.744	0.033	0.375
7 [d]	0.001	0.011	0.005	0.067	0.003	0.039

[a] The mean steady state concentration in edible fish tissue was determined to be 34 µg/kg (1.0 µg/L) and 250 µg/kg (10 µg/L).
[b] The mean steady state concentration in non-edible fish tissue was determined to be 353 µg/kg (1.0 µg/L) and 2699 µg/kg (10 µg/L).
[c] The mean steady state concentration in whole fish tissue was determined to be 186 µg/kg (1.0 µg/L) and 1492 µg/kg (10 µg/L).
[d] Percent depuration in the low and high exposure fish at day 7 were > 97 %, based on mean steady state whole fish concentration.

Table 109: Distribution of ^{14}C residues in whole fish samples, low concentration (1.0 µg/L)

Concentration of [^{14}C] residues [µg/g] (%TRR)							
Day	1	3	7	14	21	24	28
Exposure phase							
Whole fish total	0.078 (100)	0.118 (100)	0.112 (100)	0.125 (100)	0.222 (100)	0.174 (100)	0.162 (100)
Extractable	0.076 (98)	0.115 (97)	0.108 (97)	0.119 (96)	0.214 (96)	0.167 (96)	0.156 (96)
Unextractable	0.002 (2)	0.003 (3)	0.004 (3)	0.006 (4)	0.008 (4)	0.007 (4)	0.006 (4)
S-2200	0.018 (23)	0.023 (19)	0.023 (21)	0.021 (17)	0.034 (15)	0.027 (16)	0.019 (12)
10-min	n.d.	n.d.	0.009 (7.7)	0.005 (4)	0.016 (7)	0.014 (8)	0.016 (10)
11-min	0.004 (6)	0.006 (5)	n.d.	0.006 (5)	0.008 (3)	0.007 (4)	0.011 (7)
16-min [a]	0.048 (61)	0.048 (41)	0.050 (44)	0.048 (38)	0.094 (42)	0.050 (29)	0.064 (40)
17-min	n.d.	0.016 (13)	n.d.	n.d.	n.d.	0.005 (3)	0.003 (2)
19-min [a]	0.003 (4)	0.018 (15)	0.017 (15)	0.014 (11)	0.017 (7.8)	0.009 (5)	0.009 (5)
Unknown	0.003 (3)	0.004 (4)	0.010 (9)	0.024 (19)	0.045 (20)	0.054 (31)	0.034 (21)
Acetone extract	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Concentration of [^{14}C] residues [µg/g] (%TRR)							
Day	**1**	**3**	**7**	**14**	**21**	**24**	**28**
Depuration phase							
Whole fish total	0.086 (100)	0.033 (100)	0.003 (100)	-	-	-	-
Extractable	0.080 (94)	0.030 (89)	0.001 (17)	-	-	-	-
Unextractable	0.005 (6)	0.004 (11)	0.002 (83)	-	-	-	-
S-2200	n.d.	n.d.	n.d.	-	-	-	-
10-min	0.003 (4)	0.003 (9)	n.d.	-	-	-	-
11-min	0.010 (12)	0.003 (9)	n.d.	-	-	-	-
16-min [a]	0.034 (40)	0.011 (34)	n.d.	-	-	-	-
17-min	0.002 (2)	0.0003 (1)	n.d.	-	-	-	-
19-min [a]	0.011 (12)	0.003 (10)	n.d.	-	-	-	-
Unknown	0.020 (24)	0.009 (26)	0.001 (17)	-	-	-	-
Acetone extract	n.d.	n.d.	n.d.	-	-	-	-

n.d…not detectable
[a] 16-min and 19-min peaks are characterized as the glucuronic acid conjugate and /or sulphate conjugates of hydroxylated S-2200, i.e. 2-CH_2OH-S-2200, 4-OH-S-2200 and 5-CH_2OH-S-2200.

Table 110: Distribution of ^{14}C residues in whole fish samples, high concentration (10 µg/L)

Concentration of [^{14}C] residues [µg/g] (%TRR)							
Day	**1**	**3**	**7**	**14**	**21**	**24**	**28**
Exposure phase							
Whole fish total	0.835 (100)	0.927 (100)	1.017 (100)	1.577 (100)	1.367 (100)	1.458 (100)	1.651 (100)
Extractable	0.820 (98)	0.905 (98)	0.992 (97)	1.533 (97)	1.320 (97)	1.413 (97)	1.598 (97)
Unextractable	0.015 (2)	0.023 (2)	0.026 (3)	0.044 (3)	0.047 (3)	0.045 (3)	0.053 (3)
S-2200	0.191 (23)	0.220 (24)	0.241 (24)	0.246 (16)	0.275 (20)	0.232 (16)	0.255 (15)
10-min	0.036 (4)	0.030 (3)	0.030 (3)	0.047 (3)	0.036 (3)	0.023 (2)	0.050 (3)
11-min	0.054 (6)	0.037 (4)	0.032 (3)	0.045 (3)	0.051 (4)	0.035 (2)	0.045 (3)
16-min [a]	0.305 (37)	0.346 (37)	0.387 (38)	0.687 (44)	0.603 (44)	0.692 (47)	0.731 (44)
17-min	0.034 (4)	0.082 (9)	0.077 (8)	0.012 (1)	n.d.	0.038 (3)	0.036 (2)
19-min [a]	0.130 (16)	0.093 (10)	0.122 (12)	0.230 (15)	0.214 (16)	0.199 (14)	0.139 (8)
Unknown	0.079 (8)	0.097 (10)	0.103 (10)	0.264 (17)	0.141 (10)	0.195 (13)	0342 (21)
Acetone extract	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Depuration phase							
Whole fish total	0.711 (100)	0.375 (100)	0.039 (100)	-	-	-	-
Extractable	0.674 (95)	0.343 (92)	0.022 (57)	-	-	-	-
Unextractable	0.037 (5)	0.031 (8)	0.017 (43)	-	-	-	-
S-2200	n.d.	n.d.	n.d.	-	-	-	-
10-min	0.020 (3)	n.d.	0.0003 (1)	-	-	-	-
11-min	0.008 (1)	0.023 (6)	0.002 (5)	-	-	-	-
16-min [a]	0.358 (50)	0.159 (42)	0.006 (17)	-	-	-	-
17-min	0.033 (5)	0.012 (3)	n.d.	-	-	-	-
10-min [a]	0.105 (15)	0.050 (13)	0.001 (1)	-	-	-	-
Unknown	0.150 (21)	0.099 (26)	0.013 (33)	-	-	-	-
Acetone extract	n.d.	n.d.	n.d.	-	-	-	-

n.d…not detectable
[a] 16-min and 19-min peaks are characterized as the glucuronic acid conjugate and /or sulphate conjugates of hydroxylated S-2200, i.e. 2-CH_2OH-S-2200, 4-OH-S-2200 and 5-CH_2OH-S-2200.

Table 111: Summary of bioconcentration factors

	Bioconcentration factors (BCFs)					
	Low concentration (1.0 µg/L)			High concentration (10 µg/L)		
BCF	**Edible**	**Non-edible**	**Whole body**	**Edible**	**Non-edible**	**Whole body**
Total [^{14}C]Residues						
Total ^{14}C water concentration at steady state [µg S-2200 equivalent/L]	1.068	1.068	1.068	10.679	10.679	10.6679
Tissue concentration at steady state [µg S-2200 equivalent/kg]	34	353	186	250	2699	1492
$BCF_{total\ residues}$	32	331	174	23	253	140
Uptake rate constant (K_u)	15.7479	79.5256	49.0911	46.0107	70.0557	44.6165
Depuration rate constant (K_d)	0.5485	0.2776	0.3230	1.9581	0.2776	0.3229
Kinetic $BCFK_{total\ residues}$	29	286	152	23	252	138
[^{14}C]S-2200						
Measured S-2200 water concentration at steady state [µg S-2200 equivalent/L]	1.035	1.035	1.035	10.345	10.345	10.345
Tissue concentration at steady state [µg S-2200 equivalent/kg]	14	40	27	132	373	254
BCF_{S-2200}	14	39	26	13	36	25
Lipid content (wet weight) at steady state [%]	1.76	4.46	3.04	1.94	4.31	3.14
$BCF_{lipid\ total\ residues}$	18	74	57	12	59	45
$BCF_{lipid\ S-2200}$	8	9	8	7	8	8

Conclusions:

S-2200 was stable under the test conditions and reached the steady-state plateau by day 21 of exposure.

The active substance S-2200 accumulated in whole fish with steady-state BCF values in whole fish tissues of 25 and 26. BCF values for the total ^{14}C residues (TRR) were determined to be 140 and 174 for whole fish, and 253 and 331 for non-edible portions.

The uptake and depuration constants based on S-2200 were not obtained as it reached a plateau at an early stage of exposure and was not detected at all during the depuration phase. The modelled uptake rate constants (K_u) for total ^{14}C residues in whole fish tissues ranged from 44.6175 to 49.0911 per day, depuration constants (K_d) for total ^{14}C residues in whole fish tissues ranged from 0.3229 to 0.3230 per day. Greater than 95% of the ^{14}C residues were eliminated during the depuration phase (within 7 d). The depuration half-life (CT_{50}) for total ^{14}C residues was 2.1 d for whole fish.

S-2200 was extensively metabolized in fish. The major residues were the glucuronic acid conjugate and /or sulphate conjugates of hydroxylated S-2200, (i.e. 2-CH_2OH-S-2200, 4-OH-S-2200 and 5-CH_2OH-S-2200) and the active substance itself. The concentrations of the glucuronic acid conjugate and /or sulphate conjugates of hydroxylated S-2200 were determined to be between 4 and 61 % TRR in whole fish during the exposure phase,

respectively.

5.3.2 Summary and discussion of aquatic bioaccumulation

Mandestrobin has a log P_{OW} of 3.51 (mixture of both isomers) and 3.44 (*R*-isomer) and therefore a fish bioconcentration study is triggered. Based on the fish bioaccumulation study (Lentz, N.R., 2010) with *L. macrochirus* a steady-state BCF (whole fish) of 25-26 was determined, which indicate a low potential to bioaccumulate in the aquatic food chain.
The lipid corrected BCF (whole fish) of 8 was determined.

Greater than 95% of the ^{14}C residues were eliminated during the depuration phase (within 7 d). The depuration half-life (CT_{50}) for total ^{14}C residues was 2.1 d for whole fish.

S-2200 was extensively metabolized in fish. The major residues were the glucuronic acid conjugate and /or sulphate conjugates of hydroxylated S-2200, (i.e. 2-CH_2OH-S-2200, 4-OH-S-2200 and 5-CH_2OH-S-2200) and the active substance itself. The concentrations of the glucuronic acid conjugate and /or sulphate conjugates of hydroxylated S-2200 were determined to be between 4 and 61 % TRR in whole fish during the exposure phase, respectively.

The major aquatic metabolites were determined to be 2-COOH-S-2200, 5-COOH-S-2200, S-2200-OR and S-2200-ORC with an estimated (according to KOWWIN, v.1.67 estimate) log P_{OW} of 2.53, 2.88, 3.30 and 4.02, respectively. Hence, the potential of the metabolites to bioaccumulate in the aquatic food chain is considered to be low for the metabolites 2-COOH-S-2200 and 5-COOH-S-2200. The potential of the metabolites S-2200-OR and S-2200-ORC to bioaccumulate in the aquatic food chain is considered to be covered by the fish bioaccumulation study conducted with the parent compound. All metabolites making a significant contribution to total radioactive residues in the aqueous exposure medium and in fish tissues were identified and quantified. Additional studies to investigate the bioconcentration potential of metabolites, degradation and reaction products separately are therefore unnecessary and have not been conducted.

5.4 Aquatic toxicity

Standard toxicity studies on fish, aquatic invertebrates, algae and aquatic macrophytes with Mandestrobin were performed. In addition, studies with marine species were performed. Mandestrobin is toxic to the tested marine and freshwater test species. The most sensitive species is the mysid shrimp *Americamysis bahia* with an EC_{50} of 0.43 mg ai/L and a NOEC of 0.0056 mg ai/L.

Table 112: Summary of relevant information on aquatic toxicity

Method	Test organism	Test condition	Exp. time	Test conc.	Results			Reference
					Endpoint	NOEC [mg a.s./L]	EC_{50}/LC_{50} [mg a.s./L]	
Mandestrobin (S-2200)								
OECD 203, OPPTS 850.1075, EU Directive 92/69/EEC C.1	*Oncorhynchus mykiss* Rainbow trout	static	96 hr	mm	Mortality	0.57	0.94	Fournier, A., E., 2009a Report No.: ROW-0007 Study No. 13048.6622
OECD 203, OPPTS 850.1075, EU Directive 92/69/EEC C.1	*Lepomis macrochirus* Bluegill sunfish	static	96 hr	mm	Mortality	0.56	2.3	Fournier, A., E., 2009d Report No.: ROW-0008 Study No. 13048.6624
OECD 203, OPPTS 850.1075, EU Directive 92/69/EEC C.1	*Pimephales promelas* Fathead minnow	static	96 h	mm	Mortality	0.36	1.0	Fournier, A., E., 2009e Report No.: ROW-0009 Study No. 13048.6625
OPPTS 850.1075	*Cyprinodon variegatus* Sheepshead minnow	flow-through	96 hr	mm	Mortality	2.2	> 2.2	Thomas, S.T. *et al*., 2012a Report No.: ROW-0060 Study No. VP-38033
OECD 210, OPPTS 850.1400	*Pimephales promelas* Fathead minnow	flow-through	32 d	mm	Fry survival Growth	0.15	> 0.15	Michael, R.L., 2010 Report No.: ROW-0019 Study No. 13048.6626
OPPTS 850.1400	*Cyprinodon variegatus* Sheepshead minnow	flow-through	34 d	mm	Hatching success Post-hatch survival Growth	1.3 0.30 0.64	-	Minderhout, T. *et al*., 2012 Report No.: ROW-0061 Study No. VP-38042
OECD 202, OPPTS 850.1010, JMAFF 12 NohSan No. 8147, EC Guideline - Method C.2	*Daphnia magna* Water flea	static	48 h	mm	Immobilisation	0.7	1.2	Sayers, L.E., 2010a Report No.: ROW-0012 Study No. 13048.6638
OPPTS 850.1035	*Americamysis bahia* Mysid	flow-through	96 h	mm	Mortality	0.22	0.43	Thomas, S.T. *et al*., 2012a Report No.: ROW-0062 Study No. VP-38038
OPPTS 850.1025	*Crassostrea virginica* Oyster	flow-through	96 h	mm	Shell deposition	0.29	2.0	Thomas, S.T. *et al*., 2012b Report No.: ROW-0071 Study No. VP-38070
OECD 211, EC Guideline L225, C20, OPPTS	*Daphnia magna* Water flea	flow-through	21 d	mm	Immobilisation Reproduction	0.56	0.97 > 0.56	Putt, A.E., 2006b Report No.: ROW-0012

Method	Test organism	Test condition	Exp. time	Test conc.	Results			Reference
					Endpoint	NOEC [mg a.s./L]	EC_{50}/LC_{50} [mg a.s./L]	
850.1300, JMAFF 13 SeiSan No. 3986, JMAFF 12 NohSan No. 8147					Growth		> 0.56	Study No. 13048.6508
US EPA OPPTS 850.1350	*Americamysis bahia* Mysid	flow-through	36 d	mm	Immobilisation Reproduction Growth	0.0056	-	Claude, M.B. *et al*., 2012 Report No.: ROW-0063 Study No. VP-38088
US EPA 600/R-01/020	*Leptocheirus plumulosus* Marine amphipod	static-renewal	28 d	mm	Reproduction Growth	10.3 mg ai/kg	> 10.3 mg ai/kg	Thomas, S.T. *et al*., 2013a Report No.: ROW-0073 Study No. VP-38066
U.S. EPA OPPTS 850.1770 (draft, 1996), US EPA 600/R-99/064	*Hyalella azteca* Freshwater amphipod	flow-through	42 d	mm	Reproduction Growth	10.0 mg ai/kg	> 30.0 mg ai/kg	Thomas, S.T. *et al*., 2013c Report No.: ROW-0078 Study No. VP-38509
OECD 219	*Chironomus riparius* Sediment-dwelling midge	static	28 d	mm	Emergence Development	8.1	> 8.1	Picard, C.R., 2012 Report No.: ROW-0047 Study No. 13048.6671
OPPTS 850.4400, OECD 221	*Lemna gibba* Duckweed	static-renewal	7 d	mm	Growth rate Biomass	2.3 1.2	> 2.3	Jacobs, A.M. *et al*., 2012a Report No.: ROW-0065 Study No. VP-38202
S-Isomer of Mandestrobin (S-2354)								
OECD 203, OPPTS 850.1075, EU Directive 92/69/EEC C.1	*Oncorhynchus mykiss* Rainbow trout	static	96 hr	mm	Mortality	1.7	> 12	Fournier, A., E., 2009b Report No.: ROW-0011 Study No. 13048.6621
OECD 202, EC Guideline - Method C.2	*Daphnia magna* Water flea	static	48 h	mm	Immobilisation	7.3	> 14	Fournier, A., E., 2012f Report No.: ROW-0049 Study No. 13048.6706
OECD 201, EU Directive 92/69/EEC C.3	*Pseudokirchneriella subcapitata* Freshwater green alga	static	72 hr	mm	Biomass Growth rate	6.0	12 > 12	Softcheck, K.A., 2012c Report No.: ROW-0051 Study No. 13048.6705
R-Isomer of Mandestrobin (S-2167)								
OECD 203, OPPTS 850.1075, EU Directive 92/69/EEC C.1	*Oncorhynchus mykiss* Rainbow trout	static	96 hr	mm	Mortality	0.34	0.84	Fournier, A., E., 2009c Report No.: ROW-0010 Study No. 13048.6623

Method	Test organism	Test condition	Exp. time	Test conc.	Results			Reference
					Endpoint	NOEC [mg a.s./L]	EC_{50}/LC_{50} [mg a.s./L]	
OECD 202, EC Guideline - Method C.2	*Daphnia magna* Water flea	static	48 h	mm	Immobilisation	0.61	0.92	Fournier, A., E., 2012e Report No.: ROW-0048 Study No. 13048.6704
OECD 201, EU Directive 92/69/EEC C.3	*Pseudokirchneriella subcapitata* Freshwater green alga	static	72 hr	mm	Biomass Growth rate	0.13 0.26	0.38 2.2	Softcheck, K.A., 2012b Report No.: ROW-0050 Study No. 13048.6703

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Reference:	**S-2200 Technical Grade – Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions, Following OECD Guideline # 203, EC Guideline L383A, Method C.1 and OPPTS Daft Guideline 850.1075**
Author(s), year:	Fournier, Alison E., 2009a
Report/Doc. number:	Report No. ROW-0007, Study No. 13048.6622
Guideline(s):	OECD Guideline 203, US EPA OPPTS 850.1075, EC Guideline Annex V - Method C.1
GLP:	Yes
Deviations:	The protocol states that the average total length and weight of fish used for testing will be 4 to 6 cm and 0.5 to 2.0 g, respectively. The fish used during this study were an average length and weight of 3.4 cm and 0.39 g, respectively. Since the fish used for the preliminary exposure were larger in size than those used for the definitive testing and the biological response of the definitive exposure was consistent with preliminary results, this deviation is not considered to have had a negative impact on the results or interpretation of the study.
Validity:	Acceptable

Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 60020653 S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 60020652
Test species:	Rainbow trout (*Oncorhynchus mykiss*)
Number of organisms:	10 fish per replicate, 2 replicates per treatment, control and solvent control
Weight, length (mean):	0.39 g (range 0.29 – 0.51 g) and 34 mm (range 30 – 38 mm), n = 30
Loading:	0.26 g fish/L solution
Type of test, duration:	Static test, 96 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.31, 0.63, 1.3, 2.5 and 5.0 mg ai/L
Measured (mean):	- (control and solvent control), 0.33, 0.57, 1.3, 2.6 and 5.3 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Well water, total hardness: 46 mg/L as $CaCO_3$, total alkalinity: 22 mg/L as $CaCO_3$
Temperature:	13 - 15 °C (recommended 13 – 17 °C)
pH:	6.8 – 6.9 (0 h, new solution), 7.2 – 7.5 (96 h, aged solution)
O_2 content:	6.4 – 11.0 mg O_2/L (> 60% air saturation)
Light regime:	16 hours light / 8 hours darkness
Test parameters:	Mortality and sublethal effects were assessed after 0, 6, 24, 48, 72 and 96

hours.
For chemical analysis (HPLC/UV) of S-2200 in test solutions samples were taken at test initiation (0 h) and test termination (96 h) from all treatment groups and the control. Measurement of pH and dissolved oxygen concentrations were made at initiation and once daily in both vessels of each treatment. Temperature was continuously monitored throughout the study in one replicate.

Statistics: LC_{50}: Binominal probability, NOEC: Directly from raw data

Findings:

Analytical data: Since S-2200 is a mixture of two isomers, S-2354 and S-2167, the mean recovery of S-2200 was calculated by the addition of the results of the analysis for S-2354 and S-2167 from aqueous solutions.
Over the whole test period the mean measured concentrations were in the range from 91 – 110% of nominal. See Table 113

Table 113: Concentrations measured during the 96 h static acute exposure of fish to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	Measured concentration [mg ai/L] [a]							Percent of nominal [%]
	0-hour			96-hour			Mean	
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< 0.021	< 0.021	< 0.042	< 0.021	< 0.021	< 0.042	n.a.	n.a.
Solvent control	< 0.021	< 0.021	< 0.042	< 0.021	< 0.021	< 0.042	n.a.	n.a.
0.31	0.16	0.21	0.37	0.11	0.18	0.30	0.33	110
0.63	0.32	0.31	0.63	0.24	0.30	0.54	0.57	91
1.3	0.70	0.65	1.4	0.65	0.62	1.3	1.3	100
2.5	1.4	1.2	2.6	1.3	1.3	2.6	2.6	110
5.0	2.6	2.4	5.0	2.8	2.6	5.5	5.3	110

n.a…not applicable
[a] Mean measured concentrations (as S-2200) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
Concentrations expressed as less than values were below the minimum detectable limit (MDL).

Behavioural effects: Controls and concentration levels up to 0.57 mg ai/L: No sublethal effects were reported over the whole test period. At test concentration 1.3 mg ai/L following symptoms were noted after 6 hours: Lethargic behaviour, partial and complete loss of equilibrium, fish on bottom of the test vessel. After 24 hours first appearance of fish mortality. At test concentration 2.6 and 5.3 mg ai/L: 100% fish mortality after 6 hours exposure.
Thus the NOEC was 0.57 mg ai/L based on sublethal effects.

Mortality: See Table 114

Table 114: Effects on rainbow trout (*O. mykiss*) exposed to technical S-2200

S-2200 [mg ai/L] (mean measured)	Cumulative mean mortality [%]				
	6 hours	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0	0
Solvent control	0	0	0	0	0
0.33	0	0	0	0	0
0.57	0	0	0	0	0
1.3	0 [ab]	25 [bcd]	75 [bcd]	90 [a]	90 [ac]

S-2200 [mg ai/L] (mean measured)	Cumulative mean mortality [%]				
	6 hours	24 hours	48 hours	72 hours	96 hours
2.6	100	100	100	100	100
5.3	100	100	100	100	100
NOEC = 0.57 mg ai/L					
LC_{50} (96 h) = 0.94 mg ai/L (95 % C.I. 0.57 – 1.3 mg ai/L)					

[a] lethargic behaviour
[b] complete loss of equilibrium
[c] fish on bottom of the test vessel
[d] partial loss of equilibrium

Conclusion: 96 h LC_{50} = 0.94 mg ai/L
96 h NOEC = 0.57 mg ai/L
based on mean measured concentrations.

Reference:	**S-2200 Technical Grade – Acute Toxicity to Bluegill Sunfish (*Lepomis macrochirus*) Under Static Conditions, Following OECD Guideline # 203, EC Guideline L383A, Method C.1 and OPPTS Daft Guideline 850.1075**
Author(s), year:	Fournier, Alison E., 2009d
Report/Doc. number:	Report No. ROW-0008, Study No. 13048.6624
Guideline(s):	OECD Guideline 203, US EPA OPPTS 850.1075, EC Guideline Annex V - Method C.1
GLP:	Yes
Deviations:	The protocol states that the average total length and weight of fish used for testing will be 1 to 3 cm and 0.5 to 3.0 g, respectively. The fish used during this study were an average length and weight of 2.8 cm and 0.36 g, respectively. Since the fish used for the preliminary exposure were within the ranges specified in the protocol and the biological response of the definitive exposure was consistent with preliminary results, this deviation is not considered to have had a negative impact on the results or interpretation of the study. The protocol states that the test substance used is S-2200 TG (batch ST-0811G). This material was used on all occasions except the QC (quality control) preparation at the 0-hour interval. The material used for QC preparation at 0-hour only was S-2200 PAI (batch 081103G, purity 100%). Since all QC samples were adjusted for purity and QC recoveries were acceptable, this interval was not negatively impacted by this deviation.
Validity:	Acceptable

Material and methods:

Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 60020653 S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 60020652
Test species:	Bluegill sunfish (*Lepomis macrochirus*)
Number of organisms:	10 fish per replicate, 2 replicates per treatment, control and solvent control
Weight, length	0.36 g (range 0.15 – 0.54 g) and 28 mm (range 20 – 33 mm), n = 30

(mean):
Loading: 0.24 g fish/L solution
Type of test, duration: Static test, 96 hours
Applied concentrations:
Nominal: 0 (control and solvent control), 0.31, 0.63, 1.3, 2.5 and 5.0 mg ai/L
Measured (mean): - (control and solvent control), 0.29, 0.56, 1.2, 2.2 and 4.3 mg ai/L
Solvent: Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:
Water quality: Well water, total hardness: 64 mg/L as $CaCO_3$, total alkalinity: 20 mg/L as $CaCO_3$
Temperature: 21 - 22 °C (recommended 21 – 25 °C, OECD 203)
pH: 6.7 – 7.0 (0 h, new solution), 7.2 – 7.4 (96 h, aged solution)
O_2 content: 5.5 – 8.5 mg O_2/L (> 60% air saturation)
Light regime: 16 hours light / 8 hours darkness
Test parameters: Mortality and sublethal effects were assessed after 0, 6, 24, 48, 72 and 96 hours.
For chemical analysis (HPLC/UV) of S-2200 in test solutions samples were taken at test initiation (0 h) and test termination (96 h) from all treatment groups and the control. Measurement of pH and dissolved oxygen concentrations were made at initiation and once daily in both vessels of each treatment. Temperature was continuously monitored throughout the study in one replicate.
Statistics: LC_{50}: Binominal probability, probit analysis, NOEC: Directly from raw data

Findings:
Analytical data: Since S-2200 is a mixture of two isomers, S-2354 and S-2167, the mean recovery of S-2200 was calculated by the addition of the results of the analysis for S-2354 and S-2167 from aqueous solutions.
Over the whole test period the mean measured concentrations were in the range from 86 – 94% of nominal. See Table 115

Table 115: Concentrations measured during the 96 h static acute exposure of fish to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	**Measured concentration [mg ai/L] [a]**							**Percent of nominal [%]**
	0-hour			**96-hour**			**Mean**	
	S-2167	**S-2354**	**S-2200**	**S-2167**	**S-2354**	**S-2200**		
Control	< 0.01	< 0.01	< 0.02	< 0.01	< 0.01	< 0.02	n.a.	n.a.
Solvent control	< 0.01	< 0.01	< 0.02	< 0.01	< 0.01	< 0.02	n.a.	n.a.
0.31	0.14	0.16	0.30	0.12	0.16	0.28	0.29	93
0.63	0.30	0.30	0.60	0.26	0.26	0.53	0.56	89
1.3	0.64	0.64	1.3	0.57	0.56	1.1	1.2	94
2.5	1.1	1.2	2.3	1.1	1.1	2.1	2.2	89
5.0	2.1	2.2	4.3	2.1	2.2	4.3	4.3	86

n.a…not applicable
[a] Mean measured concentrations (as S-2200) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
Concentrations expressed as less than values were below the minimum detectable limit (MDL).

Behavioural effects: Control: At the end of the study (96 h) one fish died. No behavioural effects were observed.
Solvent control: At the end of the study one fish was observed to be at the bottom of the test vessel. No other behavioural effects were observed.
Concentration levels up to 0.56 mg ai/L: No sublethal effects were reported over the whole test period. At test concentration 1.2 mg ai/L two fish (10%) died at the end of the study. No behavioural effects were observed. At test concentration 2.2 following symptoms were noted after 6 hours: partial and complete loss of equilibrium, fish on bottom of the test vessel, fish at the surface of the solution, darkened pigmentation. After 6 hours first appearance of fish mortality. At the highest test concentration 100% fish mortality was observed after 6 hours exposure.
Thus the NOEC was 0.56 mg ai/L based on sublethal effects.

Mortality: See Table 116

Table 116: Effects on bluegill sunfish (*L. macrochirus*) exposed to technical S-2200

S-2200 [mg ai/L] (mean measured)	**Cumulative mean mortality [%]**				
	6 hours	**24 hours**	**48 hours**	**72 hours**	**96 hours**
Control	0	0	0	0	5
Solvent control	0	0	0	0	0 [b]
0.29	0	0	0	0	0
0.56	0	0	0	0	0
1.2	0	0	0	0	10
2.2	5	10 [abc]	30 [cd]	30 [c]	30 [ce]
4.3	100	100	100	100	100
NOEC = 0.56 mg ai/L					
LC_{50} (96 h) = 2.3 mg ai/L (95 % C.I. 1.9 – 2.8 mg ai/L)					

[a] fish at the surface of the solution
[b] fish on bottom of the test vessel
[c] partial loss of equilibrium
[d] complete loss of equilibrium
[e] darkened pigmentation

Conclusion: 96 h LC_{50} = 2.3 mg ai/L
96 h NOEC = 0.56 mg ai/L
based on mean measured concentrations.

Reference:	**S-2200 Technical Grade – Acute Toxicity to Fathead Minnow (*Pimephales promelas*) Under Static Conditions, Following OECD Guideline # 203, EC Guideline L383A, Method C.1 and OPPTS Daft Guideline 850.1075**
Author(s), year:	Fournier, Alison E., 2009e
Report/Doc. number:	Report No. ROW-0009, Study No. 13048.6625
Guideline(s):	OECD Guideline 203, US EPA OPPTS 850.1075, EC Guideline Annex V - Method C.1
GLP:	Yes
Deviations:	The protocol states that the average total length of fish used for testing will be 1 to 3 cm. The fish used during this study were an average length of 3.6 cm. Since loading rate and control performance were within protocol requirements, this deviation is not considered to have had a negative impact on the results or interpretation of the study. The protocol states that the test temperature will be maintained at 22 ± 1 °C. During this exposure, the minimum/maximum temperature ranged from 23 to 24 °C. This deviation did not have a negative impact on the results or interpretation of the study. The protocol states that the total dissolved oxygen concentrations will not be allowed to drop below 60% of saturation during the test. During the 24 hour observation interval, the dissolved oxygen levels ranged from 36 to 111% of saturation. Gentle, oil-free aeration was initiated to raise and maintain appropriated dissolved oxygen levels. During the 48 hour observation interval, dissolved oxygen levels in control replicate A dropped to 55% of saturation. The aeration was increased slightly in this replicate to correct the brief drop in dissolved oxygen. Since control performance exceeded protocol requirements, this slight deviation did not have a negative impact on the results or interpretation of the study.
Validity:	Acceptable

Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 60020653 S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 60020652
Test species:	Fathead minnow (*Pimephales promelas*)
Number of organisms:	10 fish per replicate, 2 replicates per treatment, control and solvent control
Weight, length (mean):	0.71 g (range 0.52 – 1.1 g) and 36 mm (range 32 – 41 mm), n = 30
Loading:	0.47 g fish/L solution
Type of test, duration:	Static test, 96 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.19, 0.38, 0.75, 1.5 and 3.0 mg ai/L
Measured (mean):	- (control and solvent control), 0.15, 0.36, 0.72, 1.5 and 3.0 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	

Water quality:	Well water, total hardness: 46 mg/L as $CaCO_3$, total alkalinity: 22 mg/L as $CaCO_3$
Temperature:	22 – 23 °C
pH:	6.7 – 6.8 (0 h, new solution), 7.3 – 7.4 (96 h, aged solution)
O_2 content:	3.1 – 8.4 mg O_2/L (< 60% air saturation) After 24 hours of exposure a decrease of air saturation below 60 % was observed in the controls (negative and solvent) and the treatment groups up to a test concentration of 0.75 mg ai/L. Therefore, aeration with gentle, oil-free air was initiated at 24 hours of exposure to maintain dissolved oxygen concentration at or above 60% saturation.
Light regime:	16 hours light / 8 hours darkness
Test parameters:	Mortality and sublethal effects were assessed after 0, 6, 24, 48, 72 and 96 hours. For chemical analysis (HPLC/UV) of S-2200 in test solutions samples were taken at test initiation (0 h) and test termination (96 h) from all treatment groups and the control. Measurement of pH and dissolved oxygen concentrations were made at initiation and once daily in both vessels of each treatment. Temperature was continuously monitored throughout the study in one replicate.
Statistics:	LC_{50}: Binominal probability, NOEC: Directly from raw data

Findings:

Analytical data:	Since S-2200 is a mixture of two isomers, S-2354 and S-2167, the mean recovery of S-2200 was calculated by the addition of the results of the analysis for S-2354 and S-2167 from aqueous solutions. Over the whole test period the mean measured concentrations were in the range from 81 – 100% of nominal. See Table 117

Table 117: Concentrations measured during the 96 h static acute exposure of fish to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	**Measured concentration [mg ai/L] [a]**							**Percent of nominal [%]**
	0-hour			**96-hour**			**Mean**	
	S-2167	**S-2354**	**S-2200**	**S-2167**	**S-2354**	**S-2200**		
Control	< 0.013	< 0.013	< 0.025	< 0.013	< 0.013	< 0.025	n.a.	n.a.
Solvent control	< 0.013	< 0.013	< 0.025	< 0.013	< 0.013	< 0.025	n.a.	n.a.
0.19	0.083	0.075	0.16	0.067	0.082	0.15	0.15	81
0.38	0.19	0.18	0.37	0.16	0.19	0.35	0.36	95
0.75	0.39	0.37	0.76	0.31	0.38	0.69	0.72	97
1.5	0.71	0.66	1.4	0.71	0.84	1.5	1.5	97
3.0	1.4	1.3	2.7	1.8	1.6	3.4	3.0	100

n.a…not applicable

[a] Mean measured concentrations (as S-2200) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.

Concentrations expressed as less than values were below the minimum detectable limit (MDL).

Behavioural effects:	Controls and concentration levels up to 0.36 mg ai/L: No sublethal effects were reported over the whole test period. At test concentration 0.72 mg ai/L following symptoms were noted after 6 hours: lethargic behaviour. At test concentration 1.5 mg ai/L following symptoms were noted after 6 hours: lethargic behaviour, partial and complete loss of equilibrium, fish on bottom of the test vessel. After 48 hours 100% fish mortality was observed.

Thus the NOEC was 0.36 mg ai/L based on sublethal effects.

Mortality: See Table 118

Table 118: Effects on fathead minnow (*P. promelas*) exposed to technical S-2200

S-2200 [mg ai/L] (mean measured)	Cumulative mean mortality [%]				
	6 hours	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0	0
Solvent control	0	0	0	0	0
0.15	0	0	0	0	0
0.36	0	0	0	0	0
0.72	0 [a]	0	0	0	0
1.5	30 [abcd]	95	100	100	100
3.0	100	100	100	100	100
NOEC = 0.36 mg ai/L					
LC_{50} (96 h) = 1.0 mg ai/L (95 % C.I. 0.72 – 1.5 mg ai/L)					

[a] lethargic behaviour
[b] fish on bottom of the test vessel
[c] partial loss of equilibrium
[d] complete loss of equilibrium

Conclusion: 96 h LC_{50} = 1.0 mg ai/L
96 h NOEC = 0.36 mg ai/L
based on mean measured concentrations.

Reference:	**S-2354 (S-Isomer of S-2200) – Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions, Following OECD Guideline # 203, EC Guideline L383A, Method C.1 and OPPTS Daft Guideline 850.1075**
Author(s), year:	Fournier, Alison E., 2009b
Report/Doc. number:	Report No. ROW-0011, Study No. 13048.6621
Guideline(s):	OECD Guideline 203, US EPA OPPTS 850.1075, EC Guideline Annex V - Method C.1
GLP:	Yes
Deviations:	The protocol states that the average total length and weight of fish used for testing will be 4 to 6 cm and 0.5 to 2.0 g, respectively. The fish used during this study were an average length and weight of 3.4 cm and 0.39 g, respectively. Since the water quality measurements, loading rate, and control performance were within protocol requirement, this deviation is not considered to have had a negative impact on the results or interpretation of the study.
Validity:	Acceptable

Material and methods:

Test substance: S-2354 (S-2200 *S*-isomer), purity: 99.7 %, batch: 60020653

Test species: Rainbow trout (*Oncorhynchus mykiss*)

Number of organisms: 10 fish per replicate, 2 replicates per treatment, control and solvent control

Weight, length (mean):	0.39 g (range 0.29 – 0.51 g) and 34 mm (range 30 – 38 mm), n = 30
Loading:	0.26 g fish/L solution
Type of test, duration:	Static test, 96 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 1.9, 3.8, 7.5, 15.0 and 30.0 mg ai/L
Measured (mean):	- (control and solvent control), 1.7, 3.1, 6.5, 12.0 and 9.6 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Well water, total hardness: 50 mg/L as $CaCO_3$, total alkalinity: 22 mg/L as $CaCO_3$
Temperature:	14 - 15 °C
pH:	6.7 – 6.8 (0 h, new solution), 7.1 – 7.3 (96 h, aged solution)
O_2 content:	6.4 – 10 mg O_2/L (> 60 % air saturation)
Light regime:	16 hours light / 8 hours darkness
Test parameters:	Mortality and sublethal effects were assessed after 0, 6, 24, 48, 72 and 96 hours. For chemical analysis (HPLC/UV) of S-2354 in test solutions samples were taken at test initiation (0 h) and test termination (96 h) from all treatment groups and the control. Measurement of pH and dissolved oxygen concentrations were made at initiation and once daily in both vessels of each treatment. Temperature was continuously monitored throughout the study in one replicate.
Statistics:	LC_{50}: Empirically estimated (no concentration tested resulted in ≥ 50 % mortality), NOEC: Directly from raw data
Findings:	
Analytical data:	Over the whole test period the mean measured concentrations were in the range from 32 – 88 % of nominal concentrations. Since there was undissolved test substance present at the highest treatment level tested (i.e., 30 mg ai/L nominal, 9.6 mg ai/L measured), the water accommodated fraction was siphoned and used for the exposure solution. The analytical recovery for this solution was not consistent with the range of concentrations due to the functional limit of solubility.
Behavioural effects:	Controls and concentration levels up to 3.1 mg ai/L: No sublethal effects were reported over the whole test period. At test concentration between 6.5 and 12.0 mg ai/L following symptoms were noted after 6 hours: partial and complete loss of equilibrium, lethargic behaviour, only slight gill movement, fish on bottom of test vessel. Thus the NOEC was 3.1 mg ai/L based on sublethal effects.
Mortality:	See Table 119

Table 119: Effects on rainbow trout (*O. mykiss*) exposed to S-2354 (S-2200 *S*-isomer)

S-2354 [mg ai/L] (mean measured)	**Cumulative mortality [%]**				
	6 hours	**24 hours**	**48 hours**	**72 hours**	**96 hours**
Control	0	0	0	0	0
Solvent control	0	0	0	0	0
1.7	0	0	0	0	0

S-2354 [mg ai/L] (mean measured)	Cumulative mortality [%]				
	6 hours	24 hours	48 hours	72 hours	96 hours
3.1	0	0	0	0	5 [b]
6.5	0 [a]	0 [bcd]	0 [bcd]	0 [bd]	0 [bd]
12.0	0 [b]	10 [bc]	10 [bce]	10 [bc]	20 [bc]
9.6	0 [bc]	0 [bc]	0 [bc]	0 [bc]	0 [b]
NOEC = 1.7 mg ai/L					
LC_{50} (96 h) > 12 mg ai/L					

[a] lethargic behaviour
[b] fish on the bottom of the test vessel
[c] complete loss of equilibrium
[d] partial loss of equilibrium
[e] only slight gill movement

Conclusion: 96 h LC_{50} > 12.0 mg ai/L
96 h NOEC = 1.7 mg ai/L
based on mean measured concentrations.

Reference:	**S-2167 (*R*-Isomer of S-2200) – Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions, Following OECD Guideline # 203, EC Guideline L383A, Method C.1 and OPPTS Daft Guideline 850.1075**
Author(s), year:	Fournier, Alison E., 2009c
Report/Doc. number:	Report No. ROW-0010, Study No. 13048.6623
Guideline(s):	OECD Guideline 203, US EPA OPPTS 850.1075, EC Guideline Annex V - Method C.1
GLP:	Yes
Deviations:	None
Validity:	Acceptable

Material and methods:

Test substance:	S-2167 (S-2200 *R*-isomer), purity: 100 %, batch: 60020652, CAS No.: 394657-24-0
Test species:	Rainbow trout (*Oncorhynchus mykiss*)
Number of organisms:	10 fish per replicate, 2 replicates per treatment, control and solvent control
Weight, length (mean):	0.39 g (range 0.29 – 0.51 g) and 34 mm (range 30 – 38 mm), n = 30
Loading:	0.26 g fish/L solution
Type of test, duration:	Static test, 96 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.19, 0.38, 0.75, 1.5 and 3.0 mg ai/L
Measured (mean):	- (control and solvent control), 0.17, 0.34, 0.72, 1.4 and 2.9 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Well water, total hardness: 50 mg/L as $CaCO_3$, total alkalinity: 22 mg/L as

	$CaCO_3$
Temperature:	14 – 15 °C
pH:	6.7 – 6.8 (0 h, new solution), 6.8 – 7.0 (96 h, aged solution)
O_2 content:	7.5 – 9.2 mg O_2/L (> 60 % air saturation)
Light regime:	16 hours light / 8 hours darkness
Test parameters:	Mortality and sublethal effects were assessed after 0, 6, 24, 48, 72 and 96 hours. For chemical analysis (HPLC/UV) of S-2176 in test solutions samples were taken at test initiation (0 h) and test termination (96 h) from all treatment groups and the control. Measurement of pH and dissolved oxygen concentrations were made at initiation and once daily in both vessels of each treatment. Temperature was continuously monitored throughout the study in one replicate.
Statistics:	LC_{50}: Binominal probability, NOEC: Directly from raw data
Findings:	
Analytical data:	Over the whole test period the mean measured concentrations were in the range from 89 – 96 % of nominal concentrations.
Behavioural effects:	Controls and concentration levels up to 0.34 mg ai/L: No sublethal effects were reported over the whole test period. At test concentration 0.72 mg ai/L following symptoms were noted after 6 hours: Partial and complete loss of equilibrium, lethargic behaviour, darkened pigmentation, fish on bottom of test vessel. At test concentration 1.4 and 2.9 mg ai/L: 100% fish mortality after 6 hours exposure. Thus the NOEC was 0.34 mg ai/L based on sublethal effects.
Mortality:	See Table 120

Table 120: Effects on rainbow trout (*O. mykiss*) exposed to S-2167 (S-2200 *R*-isomer)

S-2176 [mg ai/L] (mean measured)	**Cumulative mortality [%]**				
	6 hours	**24 hours**	**48 hours**	**72 hours**	**96 hours**
Control	0	0	0	0	0
Solvent control	0	0	0	0	0
0.17	0	0	0	0	0
0.34	0	0	0	0	0
0.72	0 [abcde]	5 [abd]	25	30	30
1.4	100	100	100	100	100
2.9	100	100	100	100	100
NOEC = 0.34 mg ai/L					
LC_{50} (96 h) = 0.84 mg ai/L (95 % C.I. 0.34 – 1.4 mg ai/L)					

[a] lethargic behaviour
[b] fish on the bottom of the test vessel
[c] complete loss of equilibrium
[d] partial loss of equilibrium
[e] darkened pigmentation

Conclusion:	96 h LC_{50} = 0.84 mg ai/L 96 h NOEC = 0.34 mg ai/L based on mean measured concentrations.
Reference:	**S-2200: A 96-hour flow-through acute toxicity with the Sheepshead**

	minnow (*Cyprinodon variegatus*)
Author(s), year:	Thomas, Susan T., Kendall, Timothy Z. and Gallagher, Sean P., 2012a
Report/Doc. number:	Report No. ROW-0060, Study No. VP-38033
Guideline(s):	US EPA OPPTS 850.1075 (1996)
GLP:	Yes
Deviations:	Reference substances 8993 and 8994 were used to prepare calibration standards instead of reference substances 10157 and 10158. The reason was the analyst inadvertently used an older shipment of reference materials. The older reference materials were from different lots and therefore had different expiration dates. However, the older reference materials were not yet expired and were more pure than the newer reference materials (99.7% and 100% versus 99.4% and 99.8%, respectively). This deviation from the protocol had no adverse impact upon the results or interpretation of the study.
Validity:	Acceptable

Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 60020653 S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 60020652
Test species:	Sheepshead minnow (*Cyprinodon variegatus*), juveniles
Number of organisms:	10 fish per replicate, 2 replicates per treatment, control and solvent control
Feeding:	Daily during the holding period, except during periods of fasting prior to testing, fish were fed with brine shrimp nauplii (*Artemia sp*.)
Weight, length (mean):	0.11 g (range 0.094 – 0.12 g) and 2.1 cm (range 1.8 – 2.3 cm), n = 10
Loading:	0.071 g fish/L solution
Type of test, duration:	Flow-through test, 96 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.32, 0.54, 0.90, 1.5 and 2.5 mg ai/L
Measured (mean):	- (control and solvent control), 0.29, 0.52, 0.84, 1.6 and 2.2 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2), 0.1 mL/L
Test conditions:	
Water quality:	Sand-filtered natural seawater, salinity 20 ‰, pH = 8.0
Temperature:	22 ± 2 °C
pH:	8.0 – 8.1
O_2 content:	7.4 – 7.9 mg O_2/L (> 60% air saturation)
Light regime:	16 hours light / 8 hours darkness (648 lux at the water surface)
Test parameters:	All organisms were observed periodically to determine the number of mortalities in each treatment group. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 4.5, 24, 48, 72 and 96 hours after test initiation. Temperature, dissolved oxygen, pH and salinity in the test chambers were measured. Water temperatures were within the 22 ± 2ºC range established for the test. Dissolved oxygen concentrations remained ≥ 7.4 mg/L (≥ 94%

of saturation) throughout the test. Measurements of pH ranged from 7.9 to 8.1. Salinity in the dilution water was 20 parts per thousand (‰) at test initiation and termination. Light intensity at test initiation was 648 lux at the surface of the water of one representative test chamber.
Samples were collected from each test chamber 2 days prior to the start of the test after conditioning the diluter for approximately 21 hours. Water samples also were collected from each test chamber at the beginning of the test and at 48 and 96 hours (± 1 hour) to measure concentrations of the test substance. On Day 2 of the test, it was noted that the dilution water delivery was low in the 0.54 and 1.5 mg a.i./L treatment groups at the time of analytical sample collection. Therefore samples were collected and analyzed on Day 3 to confirm the results from Day 2. All samples were collected from mid-depth, placed in glass vials, and processed immediately for analysis. Back-up samples were also collected at each sampling interval, and held under refrigerated conditions for possible future analysis.

Statistics: The absence of mortality in any of the S-2200 treatment groups during the test precluded the statistical calculation of LC_{50} values at 24, 48, 72 and 96 hours. Therefore, the LC_{50} values were estimated to be greater than the highest mean measured concentration in the study. The no-mortality concentration and the no-observed-effect concentration (NOEC) were determined by visual interpretation of the mortality and observation data.

Findings:

Analytical data: Since S-2200 is a mixture of two isomers, S-2354 and S-2167, the mean recovery of S-2200 was calculated by the addition of the results of the analysis for S-2354 and S-2167 from aqueous solutions.
Over the whole test period the mean measured concentrations were in the range from 88 and 107% of nominal. See Table 121

Table 121: Concentrations measured during the 96 h flow-through acute exposure of fish to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	Measured concentration [mg ai/L]						Mean	Percent of nominal [%]
	0-hour			96-hour				
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
0.32	0.159	0.144	0.303	0.152	0.147	0.298	0.29	91
0.54	0.252	0.231	0.483	0.252	0.241	0.493	0.52	94
0.90	0.414	0.392	0.807	0.422	0.398	0.821	0.84	93
1.5	0.717	0.697	1.41	0.694	0.654	1.35	1.6	107
2.5	1.14	1.12	2.25	1.07	1.01	2.08	2.2	88

n.a…not applicable, LOQ…Limit of quantification

Mortality and behavioural effects: No effects on survival or effects on behabivour were observed up to the highest tests concentration.

Conclusion: 96 h LC_{50} > 2.2 mg ai/L
96 h NOEC = 2.2 mg ai/L
based on mean measured concentrations.

5.4.1.2 Long-term toxicity to fish

Chronic toxicity to fish (IIA 8.2.2)

Prolonged toxicity (21 day exposure) to fish (IIA 8.2.2.1)

No study submitted. The requirement for data on the chronic effects of S-2200 on juvenile fish has been addressed by the submission of an early life stage toxicity test (ELS-test) with fathead minnow (*Pimephales promelas*) and Sheepshead minnow (*Cyprinodon variegatus*).

Fish early life stage toxicity test (IIA 8.2.2.2)

Reference:	**S-2200 Technical Grade – Early Life-Stage Toxicity Test with Fathead Minnow, *Pimephales promelas*, Following OECD Guideline #210 and OPPTS Draft Guideline 850.1400**
Author(s), year:	Michael, R. Lee, 2010
Report/Doc. number:	Report No. ROW-0019, Study No. 13048.6626
Guideline(s):	OECD Guideline 210, US EPA OPPTS 850.1400
GLP:	Yes
Deviations:	None relevant
Validity:	Acceptable
Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4 %, batch: ST-0811G
Reference substances:	S-2354 (S-2200 *S*-isomer), purity: 99.7 %, batch: 060020653 S-2167 (S-2200 *R*-isomer), purity: 100 %, batch: 060020652
Test species:	Fathead minnow (*Pimephales promelas*)
Number of organisms:	2 replicates per test concentration, control and solvent control. 30 eggs per egg incubation cup, after completion of hatch larvae were thinned to 10 individuals per replicate, 40 individuals per treatment level or controls.
Age:	Freshly fertilized eggs, 2.5 hours old
Type of test, duration:	Flow-through test, 32 days (28 days post hatch)
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.018, 0.031, 0.052, 0.088 and 0.15 mg ai/L
Measured (mean):	- (control and solvent control), 0.021, 0.030, 0.051, 0.087 and 0.15 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2), 3 µg/L
Test conditions:	
Water quality:	Well water, total hardness: 60 – 76 mg/L as $CaCO_3$, total alkalinity: 20 – 24 mg/L as $CaCO_3$, specific conductance: 420 – 450 µmhos/cm
Temperature:	24 – 26 °C
pH:	6.5 – 8.0 during the total test period
O_2 content:	6.0 – 8.9 mg O_2/L (> 60 % saturation)
Light regime:	16 hours light / 8 hours darkness, sudden transitions were avoided
Feeding	Larvae were fed of live brine shrimp nauplii (*Artemia salina*) 3 times daily beginning on day 4 post-hatch. Larvae were not fed during the final 24

hours of the test.
Residual food and fecal matter were brushed and siphoned when necessary in order to minimise microbiological growth.

Test parameters: Abnormal appearance and behaviour of larvae were assessed daily. Number of surviving larvae was estimated at least twice a week. At test termination the length and the weight were determined.
Determined endpoints were: Hatching success, overall fry survival, mean length, wet and dry weight.
Temperature, pH and dissolved oxygen concentration were measured daily.
Total hardness, alkalinity and specific conductance were measured weekly.
Analytical measurements (HPLC/UV) of S-2200 in test solutions samples were taken at 0, 4, 11, 18, 25 and 32 days.

Statistics: If control and solvent control can be pooled: t-Test
Hatching success, percentage normal larvae at hatch and percentage larval survival: Arscine square-root percentage formation
Testing for normal distribution: Shapiro Wilks' Test
Homogeneity of variance: Bartlett's Test, Levene's Test
Data met the assumptions for normal distribution and homogeneity of variance: Williams test

Findings:

Analytical data: Overall mean measured concentrations in test media were 97 - 120 % of nominal. See Table 122

Table 122: Concentrations measured in exposure solution during the early life-stage exposure of fathead minnow to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	Measured concentration [mg ai/L] [a]						Mean	Percent of nominal [%]
	Day 0			Day 32				
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< 0.0063	< 0.0063	< 0.013	< 0.0063 [a]	< 0.0063	< 0.013	n.a.	n.a.
Solvent control	< 0.0063	< 0.0063	< 0.013	< 0.0063 [a]	< 0.0063	< 0.013	n.a.	n.a.
0.018	0.011	0.010	0.021	0.011	0.011	0.022	0.021	120
0.031	0.016	0.015	0.031	0.015	0.016	0.031	0.030	98
0.052	0.026	0.025	0.051	0.026	0.026	0.052	0.051	97
0.088	0.043	0.043	0.086	0.045	0.046	0.090	0.087	99
0.15	0.078	0.074	0.15	0.078	0.076	0.15	0.15	98

n.a…not applicable
[a] Mean measured concentrations (as S-2200) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
Concentrations expressed as less than values were below the minimum detectable limit (MDL).

Biological observation: Total length of larvae: No significant differences were observed in the mean total length of the larvae from the treatment groups (26.8 – 27.66 mm) and the solvent control groups (27.7 mm).
Dry weight of larvae: No significant differences were observed in the mean dry weight of the larvae from the treatment groups (0.0455 – 0.0488 g) and the pooled control groups (0.0492 g).
Time to hatch: In controls and all treatment levels hatching completed on day 4.
Morphological and behavioural effects: Over the total test period no

morphological and behavioural effects were observed.

Effects: No significant effects on the hatching success and fry survival were observed.

Table 123: Hatching success and fry survival

S-2200 [mg ai/L] (mean measured)	Mean embryo hatching success [%]	Normal larvae at hatch (mean) [%]	Mean larval survival after 28 days post-hatch [%]
Control	89	99	95
Solvent control	90	98	95
Pooled control [a]	90	99	95
0.021	83	100	90
0.030	88	93	95
0.051	91	100	95
0.087	91	93	88
0.15	90	100	93
NOEC = 0.15 mg ai/L			
LOEC > 0.15 mg ai/L			
MATC > 0.15 mg ai/L			

[a] No statistically significant difference between dilution control and solvent control (t-Test)

Table 124: Length and Weight

S-2200 [mg a.s/L] (mean measured)	Mean length [mm] (SD) (28 d post hatch)	Mean dry weight [g] (SD) (28 d post-hatch)
Control	28.2 (0.19)	0.0505 (0.0019)
Solvent control	27.7 (0.27)	0.0479 (0.0025)
Pooled control [a]	n.a. [b]	0.0492 (0.0025)
0.021	27.6 (1.64)	0.0472 (0.0063)
0.030	27.4 (0.70)	0.0471 (0.0039)
0.051	27.5 (0.46)	0.0488 (0.0021)
0.087	26.8 (2.58)	0.0478 (0.0076)
0.15	27.5 (0.79)	0.0455 (0.0025)
NOEC = 0.15 mg ai/L		
LOEC > 0.15 mg ai/L		
EC_{50} > 0.15 mg ai/L		

[a] No statistically significant difference between dilution control and solvent control.
[b] Not applicable. Total length data was compared to the solvent control data.

Conclusion: 32 d NOEC = 0.15 mg ai/L (growth, survival)
32 d LOEC > 0.15 mg ai/L
32 d EC_{50} > 0.15 mg ai/L
based on mean measured concentrations

Reference:	**S-2200: An early life-stage test with the Sheepshead minnow (*Cyprinodon variegatus*)**
Author(s), year:	Minderhout, Tui, Kendall, Timothy Z. and Gallagher, Sean P., 2012
Report/Doc. number:	Report No. ROW-0061, Study No. VP-38042
Guideline(s):	US EPA OPPTS 850.1400 (1996)
GLP:	Yes
Deviations:	Reference substances 8993 and 8994 were used to prepare calibration standards instead of reference substances 10157 and 10158. The reason was the analyst inadvertently used an older shipment of reference materials. The older reference materials were from different lots and therefore had different expiration dates. However, the older reference materials were not yet expired and were more pure than the newer reference materials (99.7% and 100% versus 99.4% and 99.8%, respectively). This deviation from the protocol had no adverse impact upon the results or interpretation of the study.
Validity:	Acceptable

Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4 %, batch: ST-0811G
Reference substances:	S-2354 (S-2200 *S*-isomer), purity: 99.7 %, batch: 060020653 S-2167 (S-2200 *R*-isomer), purity: 100 %, batch: 060020652
Test species:	Sheepshead minnow (*Cyprinodon variegatus*), embryos (< 28 hours old)
Number of organisms:	4 replicates per test concentration, control and solvent control. 20 embryos per incubation cup, resulting in total of 80 embryos per treatment and control groups. After a 6-day embryo hatching period, the larvae were released into the test chambers, where exposure continued during a 28-day post-hatch juvenile growth period.
Biomass loading:	0.028 g fish/L (test start), 0.29 g fish/L (test termination)
Type of test, duration:	Flow-through test, 34 days (6-day hatch and 28-day post-hatch)
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.16, 0.31, 0.63, 1.3 and 2.5 mg ai/L
Measured (mean):	- (control and solvent control), 0.16, 0.30, 0.64, 1.3 and 1.9 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Sand-filtered natural seawater, salinity: 19 - 20 ‰, pH = 7.9 - 8.1
Temperature:	25 ± 2°C
pH:	7.8 – 8.0
O_2 content:	5.6 – 7.8 mg O_2/L (> 60 % saturation)
Light regime:	16 hours light / 8 hours darkness (530 lux at the surface of the water)
Feeding	Newly-hatched larvae were fed of live brine shrimp nauplii (*Artemia sp.*) 3 times per day during the first seven days post-hatch. Thereafter, they were fed live brine shrimp nauplii three times daily on weekdays and two times daily on weekends and holidays. Fish were not fed during the final 48 hours of the test. Residual food and fecal matter were brushed and siphoned when necessary

	in order to minimise microbiological growth.
Test parameters:	During the first day of exposure, embryos were observed twice for mortality and eggs with fungus. Thereafter, until hatching was complete, observations of embryo mortality and the removal of dead embryos were performed once daily. When hatching of the viable embryos in the negative control group reached >90% on Day 6 of the test, the larvae were released to their respective test chambers and the post-hatch period began. Any unhatched embryos were kept in the egg cups until they hatched and were released into the test chamber, or until death of the embryo occurred. During the 28 day post-hatch exposure period, the larvae were observed daily to evaluate the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior. From these observations, time to hatch, hatching success, and post-hatch growth and survival were evaluated. Hatching success was calculated as the percentage of embryos that hatched successfully. Post-hatch survival was calculated as the number of larvae surviving to test termination divided by the total number of embryos that hatched successfully. Post-hatch growth of the sheepshead minnows was evaluated at the conclusion of the 28-day post hatch exposure period. Total length for each surviving fish was measured to the nearest 1 mm using a metric ruler, and wet and dry weights were measured to the nearest 0.1 mg using an analytical balance. Fish were placed in an oven at approximately 60°C for approximately 48 hours to obtain dry weight data.
Statistics:	Data on time to hatch was evaluated by visual interpretation of the data. Test endpoints analyzed statistically for the juvenile fish were hatching success, larval survival and growth (total length, wet weight and dry weight). Data from the negative and solvent control groups for each parameter were compared using an appropriate test. Since no differences were detected between the two control groups ($p > 0.05$) for percent hatching success and survival endpoints, the control data were pooled for comparison among the treatment groups. However, when significant differences in growth parameters were detected between the two control groups ($p \leq 0.05$), the treatment data were compared to growth data from the negative control and solvent control separately. Hatching success and survival data were considered to be discrete-variable data, while growth data were considered continuous-variable data. Discrete-variable data were analyzed using Chi-square and Fisher's Exact test to identify treatment groups that showed a statistically significant difference ($p \leq 0.05$) from the pooled controls. All continuous-variable data were evaluated for normality using Shapiro-Wilk's test, and for homogeneity of variance using Levene's test ($p = 0.01$). Since the data passed the assumptions of normality and homogeneity, those treatments that were significantly different from the negative control or solvent control means were identified using Dunnett's one-tailed test ($p \leq 0.05$). All statistical tests were performed using a personal computer with SAS software. The results of the statistical analyses were used to aid in the determination of the NOEC, LOEC and MATC. However, scientific judgment was used to determine if statistical differences were biologically meaningful, and if the data followed a concentration-dependent response. The NOEC was

defined as the highest test concentration that produced no significant treatment-related effects on hatching success, survival or growth. The LOEC was defined as the lowest test concentration that produced a significant treatment related effect on hatching success, survival or growth. The MATC was calculated as the geometric mean of the NOEC and LOEC.

Findings:

Analytical data: Overall mean measured concentrations in test media were 76 - 102 % of nominal. See Table 125

Table 125: Concentrations measured in exposure solution during the early life-stage exposure of fathead minnow to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	Measured concentration [mg ai/L]							Percent of nominal [%]
	Day 0			Day 34			Mean	
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
0.16	0.0981	0.0984	0.196	0.0766	0.0786	0.155	0.16	100
0.31	0.155	0.156	0.310	0.171	0.173	0.344	0.30	97
0.63	0.300	0.301	0.600	0.381	0.377	0.758	0.64	102
1.3	0.571	0.539	1.11	0.766	0.778	1.54	1.3	100
2.5	0.875	0.877	1.75	0.797 [a]	0.755 [a]	1.55 [a]	1.9	76

n.a…not applicable, LOQ…Limit of quantification (0.1 mg ai/L)

[a] The mean measured concentrations are based on the measured concentrations on sampling day 0 and 48 h. At the highest test concentration no measurements of the test concentrations at sampling date 34 d (test termination) were conducted due to the 100% mortality.

Biological observation: Behavioural effects: Over the total test period morphological and behavioural effects were observed. At the test concentrations of 0.16 and 0.30 mg ai/L a few small fish were observed. At a test concentration of 0.64 mg ai/L behaviroual effects were observed, i.e. small and weak fish. At the two highest test concentrations significant behaviroual effects were observed, i.e. lethargic fish, fish lying on the bottom of the test chamber as well as small and weak fish.
See table 126.

Table 126: Hatching success, larval survival and growth

S-2200 [mg ai/L] (mean measured)	% Hatching Success	% Survival to Day 28 Post-Hatch	Growth parameters at Day 28 Post-Hatch [d]		
			Mean total length ± SD [mm]	Mean wet weight ± SD [mg]	Mean dry weight ± SD [mg]
Control	98	100	19.9 ± 0.22	99.9 ± 3.9	22.7 ± 0.87
Solvent control	96	99	20.6 ± 0.26	113.1 ± 4.1	25.7 ± 1.7
Pooled control	97	99	- [c]	- [c]	- [c]
0.16	93	99	20.5 ± 0.22	115.4 ± 4.0	26.8 ± 2.1
0.30	96	97	20.7 ± 0.26	114.3 ± 4.5	27.0 ± 1.3
0.64	91	95 *[b]	20.4 ± 0.36	114.8 ± 4.6	25.1 ± 0.84

S-2200 [mg ai/L] (mean measured)	% Hatching Success	% Survival to Day 28 Post-Hatch	Growth parameters at Day 28 Post-Hatch [d]		
			Mean total length ± SD [mm]	Mean wet weight ± SD [mg]	Mean dry weight ± SD [mg]
1.3	98	82 *[b]	18.6 ± 0.57 *	88.1 ± 7.2 *	19.4 ± 1.6 *
1.9	89 *[a]	0 *	-	-	-
NOEC (hatching success) = 1.9 mg ai/L NOEC (survival) = 1.3 mg ai/L NOEC (growth) = 0.64 mg ai/L					

* Statistically significant difference in hatching success and survival in comparison to the pooled controls ($p \leq 0.05$ using Fisher's Exact test) and in growth ($p \leq 0.05$ using Dunnett's one-tailed tests) in comparison to noth the negative control and solvent control

[a] Since the statistically significant reduction in hatching success noted in the 1.9 mg ai/L treatment group met the validity criterion set for the control group (≥ 75%), the statistically significant reduction in percent hatching success detected at the 1.9 mg a.i./L treatment group in comparison to the pooled controls was not considered biologically meaningful.

[b] Since the post-hatch survival in the 0.64 and 1.3 mg ai/L treatment groups met the validity criterion set for the control group (≥80%), the statistically significant reductions in post-hatch survival detected at the 0.64 and 1.3 mg ai/L treatment groups in comparison to the pooled controls were not considered biologically meaningful.

[c] There were statistically significant differences in growth endpoints (total length, wet and dry weights) between negative and solvent control data ($p \leq 0.05$). Therefore, the treatment data were compared to the negative control and solvent control data separately.

[d] The 1.9 mg a.i./L treatment group was excluded from analysis of growth due to 100% mortality.

Conclusion: 34 d NOEC = 0.64 mg ai/L (growth)
34 d LOEC = 1.3 mg ai/L
based on mean measured concentrations

Comment RMS: The proposed overall NOEC of 0.64 mg ai/L based on significant effects on growth. The RMS agrees on the NOEC of 0.64 mg ai/L based on effects on growth; however, the overall NOEC is proposed to be 0.3 mg ai/L based on effects on the larvae survival.

Significant effects on the survival were observed at test concentrations between 0.64 and 1.9 mg ai/L. Taking into account the effects on larval survival there is no clear dose-response considering the test concentrations between 0.16 and 0.64 mg ai/L. At the highest test concentration a 100% mortality of larvae was observed. Hence, the dose response curve is very steep. Taking into account the results of the non-GLP range-finding test similar effects were observed. At test concentrations of 0.23 and 0.75 mg ai/L effects on hatching success were 83 and 85%, respectively. The post-hatch survival was about 100% at these test concentrations. At the highest test concentration (2.5 mg ai/L) the hatching success was about 80% and the post-hatch survival was about 38% which is significant below the post-hatch survival observed in the control groups. Also in the range-finding test a comparable steep dose-response curve was observed.

The notifier proposed a NOEC of 1.9 mg ai/L and 1.3 mg ai/L based on effects on hatching success and post-hatch survival, respectively. The RMS does not agree on the proposed NOEC values. Even though the observed effects met the validity criteria set for the control group the effects should be considered biologically meaningful. First, the results are statistically significant compared to the pooled control groups. Second, the observed effects follow a dose-response indicating that the effects are treatment related.

In conclusion, the RMS is of the opinion that the NOEC considering hatching success and post-hatch survival should be 1.3 mg ai/L and 0.3 mg ai/L, respectively.

Fish life cycle test (IIA 8.2.2.3)

No study was submitted. The BCF of the active substance S-2200 is 25 – 26 and thus clearly less than 1000. In addition there was more than 95 % elimination of S-2200 residues in fish in 14 days during the depuration phase of the fish bioaccumulation study.

The acute toxicity to fish was determined to be greater than 0.1 mg/L (LC_{50} = 0.94 mg ai/L). The active substance was identified to be persistent in water and sediment (DT_{90} > 100 d) based on a worst-case DT_{90} of 1725 days. However under consideration of the low toxicity to fish and the low potential of bioaccumulation, no FLC-study is considered required.

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

Reference:	**S-2200 Technical Grade – Acute Toxicity to Water Fleas, (*Daphnia magna*), Under Static Conditions, Following OECD Guideline #202, OPPTS Draft Guideline 850.1010, The Official Journal of European Communities L383A, Method C.2 and JMAFF 12 NohSan, No. 8147 Daphnia Acute Immobilization Test (2-7-2-1) and JMAFF 13 SeiSan No. 3986**
Author(s), year:	Sayers, Lee E., 2010a
Report/Doc. number:	Report No. ROW-0013, Study No. 13048.6638
Guideline(s):	OECD Guideline 202, US EPA OPPTS 850.1010, JMAFF 12 NoHsan No. 8147, Daphnia Acute Immobilisation Test (2-7-2-1), EC Guideline Annex V - Method C.2
GLP:	Yes
Deviations:	None relevant
Validity:	Acceptable

Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 60020653 S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 60020652
Test species:	Water flea (*Daphnia magna*)
Number of organisms:	4 replicates each with 5 daphnids per treatment, control and solvent control
Age:	First instar, ≤ 24 hours old
Type of test,	Static test, 48 hours

duration:
Applied concentrations:

Nominal:	0 (control and solvent control), 0.38, 0.75, 1.5, 3.0 and 6.0 mg ai/L
Measured (mean):	- (control and solvent control), 0.35, 0.70, 1.4, 2.9 and 6.0 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Fortified well water, total hardness: 180 mg/L as $CaCO_3$, total alkalinity: 80 mg/L as $CaCO_3$, specific conductivity: 600 µmhos/cm
Temperature:	20 - 21 °C
pH:	8.1 – 8.2 (0 - 48 h)
O_2 content:	8.3 – 9.0 mg O_2/L (96 – 106 % saturation)
Light regime:	16 hours light / 8 hours darkness
Test parameters:	Immobility and sublethal effects were assessed after 0, 24 and 48 hours. For chemical analysis (LC/MS/MS) of S-2200 in the test media samples were taken at test initiation (0 h) and termination (48 h). Measurements of pH, temperature and dissolved oxygen concentrations were made at initiation and once daily. Total hardness, total alkalinity and specific conductance were measured at test initiation.
Statistics:	EC_{50}: Binominal probability, NOEC: Directly from the raw data

Findings:

Analytical data:	The overall mean measured concentration ranged from 93 – 100 % of nominal concentrations.

Table 127: Concentrations measured during the 48 h static acute exposure of daphnids to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	Measured concentration [mg ai/L] [a]							Percent of nominal [%]
	0-hour			96-hour			Mean	
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< 0.013	< 0.013	< 0.025	< 0.013	< 0.013	< 0.025	n.a.	n.a.
Solvent control	< 0.013	< 0.013	< 0.025	< 0.013	< 0.013	< 0.025	n.a.	n.a.
0.38	0.17	0.18	0.35	0.71	0.18	0.35	0.35	93
0.75	0.36	0.37	0.73	0.32	0.35	0.67	0.70	94
1.5	0.73	0.69	1.4	0.67	0.72	1.4	1.4	94
3.0	1.4	1.5	2.9	1.3	1.5	2.9	2.9	97
6.0	3.1	3.2	6.3	2.8	2.9	5.7	6.0	100

n.a…not applicable
[a] Mean measured concentrations (as S-2200) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
Concentrations expressed as less than values were below the minimum detectable limit (MDL).

Effects: After 48 hours no immobilisation was observed in the control, solvent control and in test concentrations up to 0.7 mg/L. At 1.4 and 2.9 mg/L sublethal effects (lethargy) were observed after 24 h of exposure.
Thus the NOEC was determined to be 0.70 mg ai/L and the EC_{50} was 1.2 mg ai/L.

Table 128: Effects on daphnids (*D. magna*) exposed to technical S-2200

S-2200 [mg ai/L] (mean measured)	Mean cumulative immobilised organisms [%]	
	24 hours	48 hours
Control	0	0
Solvent control	0	0
0.36	0	0
0.70	0	0
1.4	25 [a]	70 [a]
2.9	95 [a]	100
6.0	100	100
NOEC = 0.70 mg ai/L		
EC_{50} (48 h) = 1.2 mg ai/L (95 % C.I. 0.7 – 2.9 mg ai/L)		

[a] All surviving daphnids were observed to be lethargic.

Conclusion: 48 h EC_{50} = 1.2 mg ai/L
48 h NOEC = 0.7 mg ai/L
based on mean measured concentrations.

Reference:	**S-2354 (*S*-isomer of S-2200) – Acute Toxicity to Water Fleas, (*Daphnia magna*) Under Static Conditions, Following OECD Guideline #202 and The Official Journal of European Communities L 142/456, Method C.2**
Author(s), year:	Fournier, Alison E., 2012f
Report/Doc. number:	Report No. ROW-0049, Study No. 13048.6706
Guideline(s):	OECD Guideline 202 and EC Guideline Annex V - Method C.2
GLP:	Yes
Deviations:	None relevant
Validity:	Acceptable

Material and methods:

Test substance:	S-2354 (*S*-isomer of S-2200), purity: 99.7%, batch: 060020653
Test species:	Water flea (*Daphnia magna*)
Number of organisms:	4 replicates each with 5 daphnids per treatment and control
Age:	First instar, ≤ 24 hours old
Type of test, duration:	Static test, 48 hours
Applied concentrations:	
Nominal:	0 (control), 0.0.94, 1.9, 3.8, 7.5 and 15 mg/L
Measured (mean):	- (control), 0.82, 1.7, 3.5, 7.3 and 14 mg/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Fortified well water, total hardness: 190 mg/L as $CaCO_3$, total alkalinity: 96 mg/L as $CaCO_3$
Temperature:	20 - 21 °C
pH:	7.9 – 8.2 (0 – 48 h)

O_2 content:	8.4 – 9.2 mg O_2/L (99 – 107% saturation)
Light regime:	16 hours light / 8 hours darkness (light intensity 740 - 850 lux)
Test parameters:	Immobility and sublethal effects were assessed after 24 and 48 hours. For chemical analysis (HPLC) of SS-2354 in the test media samples were taken at test initiation (0 h) and termination (48 h). Measurements of pH, temperature and dissolved oxygen concentrations were made at initiation and termination of exposure.
Statistics:	EC_{50}: Spearman-Kärber Estimates, NOEC: Directly from the raw data
Findings:	
Analytical data:	The overall mean measured concentration ranged from 88 - 97% of nominal concentrations.
Effects:	After 48 hours no immobilisation was observed in the controls and in test concentrations up to 7.3 mg/L. Only 1 immobilized daphnid was observed at the highest test concentration. Sublethal effects (lethargic behaviour) were observed at the highest test concentration. Thus the NOEC was determined to be 7.3 mg/L and the EC_{50} was > 14 mg/L.

Table 129: Effects on daphnids (*D. magna*) exposed to the *S*-isomer S-2354

S-2354 [mg/L] (mean measured)	**Mean cumulative immobilised organisms [%]**	
	24 hours	**48 hours**
Control	0	0
Solvent control	0	0
0.82	0	0
1.7	0	0
3.5	0	0
7.3	0	0
14	0 [a]	5 [a]
NOEC = 7.3 mg/L		
EC_{50} (48 h) > 14 mg/L		

[a] Several daphnids were observed to be lethargic.

Conclusion:	48 h EC_{50} > 14 mg/L 48 h NOEC = 7.3 mg/L based on mean measured concentrations.

Reference:	**S-2167 (*R*-isomer of S-2200) – Acute Toxicity to Water Fleas, (*Daphnia magna*) Under Static Conditions, Following OECD Guideline #202 and The Official Journal of European Communities L 142/456, Method C.2**
Author(s), year:	Fournier, Alison E., 2012e
Report/Doc. number:	Report No. ROW-0048, Study No. 13048.6704
Guideline(s):	OECD Guideline 202 and EC Guideline Annex V - Method C.2
GLP:	Yes
Deviations:	None relevant
Validity:	Acceptable

Material and methods:	
Test substance:	S-2167 (*R*-isomer of S-2200), purity: 100%, batch: 060020652
Test species:	Water flea (*Daphnia magna*)
Number of organisms:	4 replicates each with 5 daphnids per treatment and control
Age:	First instar, ≤ 24 hours old
Type of test, duration:	Static test, 48 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.078, 0.16, 0.31, 0.63, 1.3 and 2.5 mg/L
Measured (mean):	- (control and solvent control), 0.062, 0.14, 0.29, 0.61, 1.2 and 2.5 mg/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Fortified well water, total hardness: 190 mg/L as $CaCO_3$, total alkalinity: 95 mg/L as $CaCO_3$
Temperature:	20 - 21 °C
pH:	8.1 – 8.2 (0 – 48 h)
O_2 content:	8.3 – 9.2 mg O_2/L (98 – 107% saturation)
Light regime:	16 hours light / 8 hours darkness (light intensity 600 – 750 lux)
Test parameters:	Immobility and sublethal effects were assessed after 24 and 48 hours. For chemical analysis (HPLC) of SS-2167 in the test media samples were taken at test initiation (0 h) and termination (48 h). Measurements of pH, temperature and dissolved oxygen concentrations were made at initiation and termination of exposure.
Statistics:	EC_{50}: Spearman-Kärber Estimates, NOEC: Directly from the raw data
Findings:	
Analytical data:	The overall mean measured concentration ranged from 80 - 99 % of nominal concentrations.
Effects:	After 48 hours no immobilisation was observed in the controls and in test concentrations up to 0.61 mg/L. At the highest test concentration of 2.5 mg/L the immobilisation was 100%. Sublethal effects (lethargic behaviour) were observed at 1.2 mg/L treatment group only. Thus the NOEC was determined to be 0.61 mg/L (based on mortality and behavioural effects) and the EC_{50} was 0.92 mg/L. See Table 130.

Table 130: Effects on daphnids (*D. magna*) exposed to the *R*-isomer S-2167

S-2167 [mg/L] (mean measured)	**Mean cumulative immobilised organisms [%]**	
	24 hours	**48 hours**
Control	0	0
Solvent control	0	0
0.062	0	0
0.14	0	0
0.29	0	0
0.61	0	0
1.2	75 [a]	90 [a]
2.5	100	100
NOEC = 0.61 mg/L		
EC_{50} (48 h) = 0.92 mg/L (95 % C.I. 0.84 – 1.0 mg/L)		

[a] One daphnid was observed to be lethargic.

Conclusion: 48 h EC_{50} = 0.92 mg/L
48 h NOEC = 0.61 mg/L
based on mean measured concentrations.

Reference:	**S-2200: A 96-hour flow-through acute toxicity test with the saltwater mysid (*Americamysis bahia*)**
Author(s), year:	Thomas, Susan T., Kendall, Timothy Z. and Krueger, Henry O., 2012
Report/Doc. number:	Report No. ROW-0062, Study No. VP-38038
Guideline(s):	US EPA OPPTS 850.1035 (1996)
GLP:	Yes
Deviations:	Reference substances 8993 and 8994 were used to prepare calibration standards instead of reference substances 10157 and 10158. The reason was the analyst inadvertently used an older shipment of reference materials. The older reference materials were from different lots and therefore had different expiration dates. However, the older reference materials were not yet expired and were more pure than the newer reference materials (99.7% and 100% versus 99.4% and 99.8%, respectively). This deviation from the protocol had no adverse impact upon the results or interpretation of the study.
Validity:	Acceptable

Material and methods:

Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 60020653 S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 60020652
Test species:	Saltwater mysid (*Americamysis bahia*), juveniles (< 24 hours old)
Number of organisms:	10 mysids per replicate, 2 replicates per treatment, control and solvent control
Feeding:	Daily during the holding period and twice daily during the test, mysids were fed with brine shrimp nauplii (*Artemia sp*.)
Type of test, duration:	Flow-through test, 96 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.063, 0.13, 0.25, 0.50 and 1.0 mg ai/L

Measured (mean):	- (control and solvent control), 0.052, 0.12, 0.22, 0.49 and 0.92 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2), 0.1 mL/L
Test conditions:	
Water quality:	Sand-filtered natural seawater, salinity 20 ‰, pH = 8.0
Temperature:	25 ± 1 °C
pH:	7.9 – 8.0
O_2 content:	6.2 – 7.8 mg O_2/L (> 60% air saturation)
Light regime:	16 hours light / 8 hours darkness (536 lux at the water surface)
Test parameters:	All organisms were observed periodically to determine the number of mortalities in each treatment group. The criteria for death include lack of movement, absence of respiratory movements, and lack of reaction to gentle prodding. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 3.5, 24, 48, 72 and 96 hours after test initiation. Temperature, dissolved oxygen, pH and salinity in the test chambers were measured. Water temperatures were within the 25 ± 2ºC range established for the test. Dissolved oxygen concentrations remained ≥6.2 mg/L (≥85% of saturation) throughout the test. Measurements of pH ranged from 7.9 to 8.0 during the test. Salinity of the dilution water at test initiation and termination was 20‰. Light intensity at test initiation was 536 lux at the surface of the water of one representative test chamber. Samples were collected from each test chamber 6 days prior to the start of the test after conditioning the diluter for approximately 21 hours. Water samples also were collected from each test chamber at the beginning of the test and at 48 and 96 hours (± 1 hour) to measure concentrations of the test substance. The samples were collected from mid-depth, placed in glass vials, and processed immediately for analysis. Back-up samples were also collected at each sampling interval, and held under refrigerated conditions for possible future analysis.
Statistics:	The mortality data were analyzed using the computer program of C. E. Stephan. The program was designed to calculate the LC_{50} value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation. In this study, non-linear interpolation with binomial probability was used to calculate the 48, 72 and 96 hour LC_{50} values and the 95% confidence intervals. Due to the method used to calculate the 96 hour LC_{50} value, the slope of the dose response curve could not be calculated. Since there was <50% mortality at 24 hours, the 24 hour LC_{50} value, as well as the no-mortality concentration and NOEC, were determined by visual interpretation of the mortality and observation data.
<u>Findings:</u>	
Analytical data:	Since S-2200 is a mixture of two isomers, S-2354 and S-2167, the mean recovery of S-2200 was calculated by the addition of the results of the analysis for S-2354 and S-2167 from aqueous solutions. Over the whole test period the mean measured concentrations were in the range from 83 and 98% of nominal.

Table 131: Concentrations measured during the 96 h flow-through acute exposure of saltwater mysids to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	Measured concentration [mg ai/L]							Percent of nominal [%]
	0-hour			96-hour			Mean	
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
0.063	0.0219	0.0184	0.0403	0.0297	0.0300	0.0597	0.052	83
0.13	0.0531	0.0526	0.106	0.0614	0.0612	0.123	0.12	92
0.25	0.110	0.105	0.215	0.119	0.116	0.234	0.22	88
0.50	0.219	0.228	0.467	0.247	0.244	0.491	0.49	98
1.0	0.469	0.446	0.916	0.467 [a]	0.465 [a]	0.932	0.92	92

n.a…not applicable, LOQ…Limit of quantification (0.04 mg ai/L)
[a] The mean measured concentrations are based on the measured concentrations on sampling day 0 and 48 h. At the highest test concentration no measurements of the test concentrations at sampling date 96 h (test termination) were conducted due to the 100% mortality.

Mortality and behavioural effects: See Table 132

Table 132: Effects on mysids (*Americamysis bahia*) exposed to technical S-2200

S-2200 [mg ai/L] (mean measured)	Mean cumulative immobilised organisms [%]			
	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0
Solvent control	0	0	0	0
0.052	0	0	0	0
0.12	0	0	0	10
0.22	0	0	0	0
0.49	0	0	40 [a]	65 [a]
0.92	0	100	100	100

[a] lethargic behaviour

Conclusion: 96 h LC_{50} = 0.43 mg ai/L (95% C.I. 0.22 – 0.49 mg ai/L)
96 h NOEC = 0.22 mg ai/L
based on mean measured concentrations.

Reference:	**S-2200: A 96-hour shell deposition test with the Eastern Oyster (*Crassostrea virginica*)**
Author(s), year:	Thomas, Susan T., Kendall, Timothy Z. and Gallagher, Sean P., 2012b
Report/Doc. number:	Report No. ROW-0071, Study No. VP-38070
Guideline(s):	US EPA test guideline OPPTS 850.1025 (1996)
GLP:	Yes
Deviations:	Reference substances 8993 and 8994 were used to prepare calibration standards instead of reference substances 10157 and 10158. The reason was the analyst inadvertently used an older shipment of reference materials. The older reference materials were from different lots and therefore had different expiration dates. However, the older reference materials were not yet expired and were more pure than the newer reference materials (99.7% and 100% versus 99.4% and 99.8%, respectively). This deviation from the protocol had no adverse impact upon the results or interpretation of the study. It was noted on the morning of October 18, 2011 (Day 1), that the algae feed suspension was not being delivered to the 2.5 mg a.i./L mixing chamber. The protocol indicated that if a diluter malfunction was detected or suspected, a water sample would be collected for test substance analysis. This was inadvertently not done at the time the malfunction was noted. The reason was biologist oversight. The malfunction was corrected immediately upon detection. Based upon a check of the test system on the afternoon of Day 0, the algae feed delivery represents a minimal portion (~5%) of the dilution water volume being delivered and therefore the impact on the test concentration in the test chambers was minimal. Also, it was noted during the study that the 2.5 mg a.i./L treatment group has excess food present in the test chambers due to lack of feeding, therefore the temporary interruption in food delivery had no impact on the growth of the oysters. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.
Validity:	Acceptable

Material and methods:

Test substance:	S-2200 technical grade, purity: 93.4 %, batch: ST-0811G
Reference substances:	S-2354 (S-2200 *S*-isomer), purity: 99.7 %, batch: 060020653 S-2167 (S-2200 *R*-isomer), purity: 100 %, batch: 060020652
Test species:	Eastern oysters (*Crassostrea virginica*)
Number of organisms:	20 oysters per treatment, control and solvent control.
Age:	Oysters were of similar age (mean valve height relative to standard deviation was 9.1%), mean lenght of 38.8 ± 3.4 mm, range: 32.5 – 43.2 mm (n = 20) Prior to testing, recently deposited shell was removed by grinding the periphery of the oysters using a electric grinder.
Type of test, duration:	Flow-through test, 96 hours
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.31, 0.63, 1.3, 2.5 and 5.0 mg ai/L
Measured (mean):	- (control and solvent control), 0.29, 0.60, 1.2, 2.1 and 4.2 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)

Test conditions:

Water quality:	Sand-filtered natural seawater, salinity: 20 ‰, pH = 8.0
Temperature:	20 ± 2 °C
pH:	7.9 – 8.0 (0 - 96 h)
O_2 content:	6.1 – 7.6 mg O_2/L (> 60 % saturation)
Light regime:	16 hours light / 8 hours darkness (393 lux at the water surface)
Feeding:	During the exposure, the oysters received supplemental feedings of marine microalgae, approximately 5.8 x 10^9 cells/oyster/day.
Test parameters:	All organisms were observed periodically for general health and to determine the number of mortalities in each treatment group. Oysters having open shells and not responding to gentle prodding were considered dead. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 5, 24, 48, 72 and 96 hours after test initiation. At the end of the test, the longest finger of new shell growth on each oyster was measured to the nearest 0.1 mm using calipers. Temperature, dissolved oxygen, pH and salinity in the test chambers were measured. Water temperatures were within the 20 ± 2 ºC range established for the test. Dissolved oxygen concentrations remained ≥ 6.1 mg/L (≥ 76% of saturation) throughout the test. Measurements of pH ranged from 7.9 to 8.0. Salinity in the test chambers was 20 parts per thousand (‰) at test initiation and termination. Light intensity at test initiation was 393 lux at the surface of the water of one representative test chamber. Nominal concentrations selected for use in this study were 0.31, 0.63, 1.3, 2.5 and 5.0 mg a.i./L. During the course of the test, the appearance of the solution at these nominal concentrations was observed in the test chambers, as well as in the diluter mixing chambers, where test substance stocks and dilution water were combined prior to delivery to the test chambers. The solutions in the mixing chambers of the negative and solvent controls and at 0.31 to 2.5 mg a.i./L treatment levels appeared clear and colorless at test initiation. A very slight amount of precipitate was noted in the mixing chamber at the 5.0 mg a.i./L treatment level at test initiation. At test termination, the solutions in the mixing chambers for the controls and the 0.31 to 1.3 mg a.i./L treatment groups appeared clear and colorless with no visible precipitate, while the 2.5 and 5.0 mg a.i./L treatment groups had precipitate in the mixing chambers, increasing in amount with increasing concentration. At test initiation, all test solutions in all the test chambers appeared clear and colorless with no visible precipitate. At test termination, all solutions in the test chambers at 0.31 to 1.3 mg a.i./L treatment levels appeared clear and light green in color due to algal feed with no visible sign of precipitate. Solutions in the test chambers at the 2.5 and 5.0 mg a.i./L treatment levels also appeared clear but had a green color, increasing in intensity with increasing concentration due to algal feed, with no visible precipitate. Due to the presence of a precipitate in the 5.0 mg a.i./L mixing chamber and the possibility of undissolved material in the test chamber, water samples collected from this treatment level were centrifuged prior to analysis at each interval. Water samples from the 2.5 mg a.i./L treatment group were also centrifuged prior to analysis on Day 4, due to the

observation of precipitate in the mixing chamber.

Statistics: The shell deposition data from the negative control and solvent control were compared using an appropriate statistical test. There were no significant differences between the control groups ($p > 0.05$), however, growth inhibition was evaluated on the basis of the negative control data. The EC_{50} value, the concentration of test substance that would inhibit shell deposition by 50% relative to the negative control, was calculated using linear interpolation.
The shell deposition data were evaluated for normality and homogeneity of variance using the Chi-Square test and Bartlett's test, respectively. The data passed the assumptions of normality but did not meet the assumptions of the homogeneity of variances. The data was corrected using a square root transformation and assumptions of homogeneity were met. The data in the treatment groups were compared to the negative control data using a Dunnett's t-test to identify any significant differences. The EC_{50} was calculated using means corrected by square root transformation. The no-observed-effect concentration (NOEC) was determined from the statistical analysis of the data and an assessment of the concentration-response pattern. Statistical analyses were conducted using TOXSTAT® computer software.

Findings:

Analytical data: The overall mean measured concentration ranged from 84 - 95 % of nominal concentrations.

Table 133: Concentrations measured in exposure solution during the acute exposure of eastern oyster to S-2200 technical grade

S-2200 [µg ai/L] (nominal)	**Measured concentration [mg ai/L]**							**Percent of nominal [%]**
	Day 0			**Day 4**			**Mean**	
	S-2167	**S-2354**	**S-2200**	**S-2167**	**S-2354**	**S-2200**		
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
0.31	0.127	0.117	0.244	0.153	0.160	0.313	0.29	94
0.63	0.278	0.284	0.562	0.301	0.306	0.607	0.60	95
1.3	0.604	0.615	1.22	0.628	0.619	1.25	1.2	92
2.5	1.04	1.08	2.13	1.19	1.17	2.36	2.1	84
5.0	2.18	2.22	4.39	2.18	2.10	4.28	4.2	84

n.a…not applicable, LOQ…Limit of quantification

Effects: No mortality or adverse effects were observed among oysters at any of the treatment levels.
Information on mean shell deposition and inhibition of shell growth see Table

Table 134: Effects on oysters (*Crassostrea virginica*) exposed to S-2200

S-2200 [mg ai/L] (mean measured)	**Morality [%]**	**Mean shell deposition at 96 h ± SD [mm]**	**Shell growth inhibition relative to control [%]**
Control	0	5.0 ± 1.9	-
Solvent control	0	4.1 ± 1.8	17
0.29	0	4.3 ± 2.0	14
0.60	0	3.4 ± 1.8 *	31
1.2	0	3.0 ± 1.4 *	40
2.1	0 [ab]	1.5 ± 1.2 *	69
4.2	0 [ac]	0.29 ± 0.62 *	94
96 h EC_{50} = 2.0 mg ai/L (95% C.I. 1.7 – 2.6 mg ai/L) based on shell growth NOEC = 0.29 mg ai/L			

SD…Standard deviation
* Statistically significant compared to the negative control using a Dunnett'st-test ($p \leq 0.05$)
[a] Test solution appreared dark green in color due to lack of feeding
[b] At test end 12 of 20 organisms did not apprear to be open and feeding
[c] At test end 20 of 20 organisms did not appear to be open and feeding

Conclusion: 96 h EC_{50} = 2.0 mg ai/L
96 h NOEC = 0.29 mg ai/L
based on mean measured concentrations

5.4.2.2 Long-term toxicity to aquatic invertebrates

Reference:	**S-2200 Technical Grade – Full Life-Cycle Toxicity Test with Water Fleas, *Daphnia magna* Under Flow-Through Conditions, Following OPPTS Draft Guideline 850.1300, OECD Guideline #211, The Official Journal of the European Communities L225, Method C.20, JMAFF 12 NohSan, No. 8147 *Daphnia spp*. Reproduction Toxicity Studies (2-7-2-3) and JMAFF 13 SeiSan No. 3986**
Author(s), year:	Sayers, Lee E., 2010
Report/Doc. number:	Report No. ROW-0020, Study No. 13048.6639
Guideline(s):	OECD Guideline 211, OPPTS Draft Guideline 850.1300, EC Guideline L225, Method C.20, JMAFF 12 NohSan Guideline No. 8147, JMAFF 13 SeiSan Guideline No. 3986
GLP:	Yes
Deviations:	The protocol states that samples will be removed from each control and test concentration on days 0, 8, 10, 14, 16, 20 and 21. During this exposure, 100% immobilisation was observed in the 1.2 mg ai/L (nominal) treatment level as of day 3 of exposure. Samples were removed from the aged solution at this concentration at day 8 but additional solutions at this concentration were not prepared or analysed at subsequent intervals. This deviation did not have a negative impact on the results or interpretation of the study. The protocol states that dissolved oxygen levels will not be allowed to drop below 60% of saturation for the duration of the study. At several intervals during this study, dissolved oxygen levels in aged test solutions of the solvent control, 0.075, 0.15, 0.30 and 0.60 mg ai/L (nominal) treatment levels were found to be below 60% of saturation. Since these deviations were observed in the aged test solutions, which were renewed at 48-hour intervals, and no adverse effects were observed throughout the study at these concentrations, this deviation did not have a negative impact on the results or interpretation of the study.
Validity:	Acceptable

<u>Material and methods:</u>	
Test substance:	S-2200 technical grade, purity: 93.4 %, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 60020653 S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 60020652
Test species:	Water flea (*Daphnia magna*)
Number of organisms:	10 replicates per treatment group and controls, each with 10 daphnids
Age:	First instar, < 24 hours old
Type of test, duration:	Flow-through test, 21 d
<u>Applied concentrations:</u>	
Nominal:	0 (control and solvent control), 0.075, 0.15, 0.30, 0.60 and 1.2 mg ai/L

Measured (mean):	- (control and solvent control), 0.076, 0.15, 0.31, 0.56 and 1.4 mg ai/L
Solvent:	Acetone (CAS No. 67-64-1)
Test conditions:	
Water quality:	Fortified well water, hardness: 180 – 190 mg/L as $CaCO_3$, alkalinity: 86 – 100 mg/L as $CaCO_3$, specific conductivity: 600 – 650 µmhos/cm
Temperature	19 – 21 °C
pH	7.5 – 9.0
O_2 content:	New solution: 8.4 – 11 mg O_2/L (> 60 % saturation) Old solution: 3.8 – 9.7 mg O_2/L (40 – 110 % saturation)
Light regime:	16 hours light / 8 hours darkness
Feeding	Daphnids were fed daily with 200 µL of algal suspension (*Ankistrodesmus falcatus*, 4 x 10^7 cells/mL), and 50 µL mL of a yeast, cereal leaves and digested flaked fish food suspension (equivalent to 0.42 mg carbon/daphnid/day).
Test parameters:	At each renewal period (test initiation and at 48-hour intervals thereafter), freshly prepared test solution was added so a second set of clean beakers and daphnids were carefully transferred from the aged solution into the freshly prepared test solutions. Parent mobility, mortality and abnormal behaviour were observed daily. Reproduction (mean time to first brood, age at first brood, offspring per surviving parental) were observed on day 7 and three times per week through day 21. At test termination body length and parental body mass (dry weight) were reported. For chemical analysis (LC/MS/MS) of S-2200 in the newly prepared test media samples were taken on days 0, 8, 14 and 20 from each test concentration. Additionally, aged test solutions were sampled and analysed on test days 2, 10, 16 and 21. Measurements of pH, dissolved oxygen and temperature in each test and control solution were made at initiation and end of each renewal period. Total hardness, alkalinity and specific conductance were measured and recorded in the freshly prepared solutions of the highest available nominal test concentration and the control at test initiation and weekly thereafter.
Statistics:	Comparison of the number of surviving daphnids in the control to each mean measured concentration: Fisher's Exact Test Comparison of the performance of the control organisms with that of the solvent control organisms: Student's t-Test Chi-Square Test for normality was used to compare the observed sample distribution with a normal distribution for all endpoints. Check of homogeneity of variance (reproduction, length and weight): Bartlett's Test Normal distribution and homogeneity of variance (reproduction and growth): William's Test NOEC and EC_{50} were empirically estimated. TOXSTAT® version 3.5 was used to perform the statistical computations.
Findings:	
Analytical data:	The mean measured concentrations ranged from 94 - 120% of nominal concentrations.

Table 135: Concentrations measured in exposure solution during the 21-day chronic exposure of daphnids to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	Measured concentration [mg ai/L] [a]							Percent of nominal [%]
	Day 0 (new)			Day 21 (aged)			Mean	
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< 0.005	< 0.005	< 0.01	< 0.005	< 0.005	< 0.01	n.a.	n.a.
Solvent control	< 0.005	< 0.005	< 0.01	< 0.005	< 0.005	< 0.01	n.a.	n.a.
0.075	0.039	0.042	0.082	0.038	0.036	0.074	0.076	100
0.15	0.077	0.079	0.016	0.073	0.076	0.15	0.15	98
0.30	0.16	0.17	0.32	0.16	0.17	0.33	0.31	100
0.60	0.20	0.21	0.41	0.36	0.37	0.73	0.56	94
1.2	0.70	0.75	1.4	[b]	[b]	[b]	1.4	120

n.a…not applicable
[a] Mean measured concentrations (as S-2200) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
Concentrations expressed as less than values were below the minimum detectable limit (MDL).
[b] Samples were not analysed due to 100% immobilisation in this treatment group.

Biological observation: In the dilution water control and solvent control daphnids released their first brood of offspring on test day 9 and day 8, respectively. In the treatment groups first brood release occurred on test day 7, 7, 9 and 8 in the 0.076, 0.15, 0.31 and 0.56 mg ai/L treatment levels, respectively. Due to 100% immobilisation by test day 3, no offspring were released in the 1.4 mg ai/L treatment level.

Effects: See Table 136

Table 136: Summary of effects of long-term exposure of S-2200 on *Daphnia magna*

S-2200 [mg ai/L] (nom)	S-2200 techn. [mg ai/L] (mm)	Mean percent survival at day 21 [%]	Mean number of offspring per surviving female at day 21 (SD)	Mean (SD) Dry weight of parent after 21 d [mg]	Mean (SD) body length of parent after 21 d [mm]
Control	Control	90	135 (10)	1.09 (0.08)	4.86 (0.07)
Solvent control	Solvent control	90	139 (13)	1.14 (0.13)	4.81 (0.12)
Pooled control [a]	Pooled control	90	137 (11)	1.12 (0.11)	4.83 (0.10)
0.075	0.076	90	147 (16)	1.03 (0.13)	4.89 (0.06)
0.15	0.15	90	138 (9)	1.05 (0.07)	4.84 (0.06)
0.30	0.31	100	138 (11)	1.12 (0.08)	4.86 (0.07)
0.60	0.56	100	128 (21)	1.13 (0.09)	4.86 (0.11)
1.2	1.4	0 [b]	n.a.	n.a.	n.a.
NOEC (based on mean measured)		0.56 mg ai/L	0.56 mg ai/L	0.56 mg ai/L	0.56 mg ai/L
LOEC (based on mean measured)		1.4 mg ai/L	> 0.56 mg ai/L	> 0.56 mg ai/L	> 0.56 mg ai/L
EC_{50} (based on mean measured)		0.97 mg ai/L	> 0.56 mg ai/L	> 0.56 mg ai/L	> 0.56 mg ai/L
MATC (based on mean measured)		0.89 mg ai/L			

S.D…Standard Deviation, n.a…not applicable, mm…mean measured, nom…nominal
[a] No statistically significant difference between control and solvent control.
[b] Significantly reduced compared to the pooled control, based on the Fisher Exact Test. This treatment level was excluded from further statistical analysis (i.e. reproduction and growth) due to the survival effect observed.

Conclusion:	NOEC = 0.56 mg ai/L (immobilisation, reproduction, growth, weight and length) LOEC > 0.56 mg ai/L (reproduction, growth, weight and length) EC_{50} = 0.97 mg ai/L (immobilisation) EC_{50} > 0.56 mg ai/L (reproduction, growth, weight and length) based on mean measured concentrations

Reference:	**S-2200: A flow-through life-cycle toxicity test with the saltwater mysid (*Americamysis bahia*)**
Author(s), year:	Claude, Mary Beth, Kendall, Timothy Z., Gallagher, Sean P. and Krueger, Henry O., 2012
Report/Doc. number:	Report No. ROW-0063, Study No. VP-38088
Guideline(s):	US EPA OPPTS 850.1350 (1996)
GLP:	Yes
Deviations:	On day 35 of the test, a male mortality occurred in compartment 1 replicate A in the 100 µg a.i./L treatment group and was inadvertently not replaced with an extra male. The protocol states that additional male mysids will be maintained in a separate test compartment of each replicate test chamber and that these additional males will be used to replace dead males in the same replicate during the exposure. The reason for this deviation was biologist error. This occurred the day before the study was terminated and the replicate had already demonstrated sufficient reproduction data. Reproductive data from the remaining compartments in replicate A as well as the remaining replicates in the 100 µg a.i./L treatment group provided sufficient information for statistical analysis. Therefore, this deviation from the protocol had no adverse impact upon the results or interpretation of the study. On Day 27 of the study, five young G2 mysids were recorded as being present in Compartment 4, Replicate A of the 25 µg a.i./L treatment group. The observations of these young mysids were inadvertently not recorded at that time. The protocol states that observations will be made daily for the G1 and G2 mysids. The reason for this deviation was biologist error. It can be concluded that since five G2 mysids were initiated on December 14, 2011, the 5 young mysids observed in this compartment must have been observed to be alive. While observations were not made the day they were initiated, observations on the following day indicated that four mysids appeared normal and one had died, and still provided sufficient information to aid in the determination of sub-lethal effects for the study. Therefore, this deviation from the protocol had no adverse impact upon the results or interpretation of the study.
Validity:	Acceptable

Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4 %, batch: ST-0811G
Reference substances:	S-2354 (S-2200 *S*-isomer), purity: 99.7 %, batch: 060020653 S-2167 (S-2200 *R*-isomer), purity: 100 %, batch: 060020652

Test species:	Saltwater mysids (*Americamysis bahia*), neonates (< 24 hours old)
Number of organisms:	4 replicates per test concentration, control and solvent control. 15 neonate mysids per replicate, resulting in total of 60 neonates per treatment and control groups. On Day 14 of the test, after mysids attained sexual maturity, male and female adults were paired in each treatment and control group, with a maximum of five reproductive pairs per replicate. Reproduction of the paired mysids was monitored through termination on Day 36.
Type of test, duration:	Flow-through test, 36 days
Applied concentrations:	
Nominal:	0 (control and solvent control), 6.3, 13, 25, 50 and 100 µg ai/L
Measured (mean):	- (control and solvent control), 5.6, 11, 24, 49 and 84 µg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Water quality:	Sand-filtered natural seawater, salinity: 19 - 20 ‰, pH = 8.0 – 8.1
Temperature:	25 ± 2°C
pH:	7.9 – 8.2
O_2 content:	5.8 – 7.4 mg O_2/L (> 60 % saturation)
Light regime:	14 hours light / 10 hours darkness (115 lux at the surface of the water)
Feeding	Daily during the holding period and up to four times daily during the test, mysids were fed with brine shrimp nauplii (*Artemia sp*.). Daily, the mysids were fed the enriched brine shrimp for one of the daily feedings during the test. Mysid food was also periodically supplemented with *Skeletonema costatum*, a saltwater alga.
Test parameters:	Observations of the survival and behavior of each first-generation mysid were made daily throughout the test. The criteria for death included lack of movement, absence of respiratory movements, and lack of reaction to gentle prodding. At pairing on Day 14, the sex and maturity of each mysid was determined by microscopic examination, and when possible, five male/female pairs were assigned to reproductive compartments in each replicate test chamber, with one pair per compartment. Any immature mysids or extra females were discarded at this time. Any sexually mature males remaining after pairing were maintained in a separate compartment within the respective replicate test chamber. If a male in a reproductive compartment died, it was replaced with a male from the pool of males maintained in the same replicate, if available. Following pairing, second-generation mysids produced in each compartment were counted, recorded and removed daily. Second-generation mysids were also observed for abnormal development and aberrant behavior. The test was terminated on Day 36, which was at least seven days past the median time of first brood release for the negative and solvent controls (Day 27). At test termination, the sex of each surviving first-generation mysid was confirmed and the total length of each mysid was measured using calipers. The mysids then were placed in a drying oven at approximately 60°C for approximately 117 hours to obtain dry weight data. When available, one group per test concentration of G_2 mysids ($n \geq 5$) produced during the reproductive phase were maintained under their

respective test conditions and observed for mortality, signs of toxicity, appearance, and behavior for 96 hours post release. Offspring produced on the same day were pooled from different replicates per concentration to obtain the required number of animals.

Temperature, dissolved oxygen, and pH were measured in each test chamber at the beginning and end of the test, and approximately weekly during the test, with measurements typically rotating among the replicates in each group at each measurement interval. In addition, temperature was monitored continuously and salinity was measured daily in one negative control test chamber. After mysids attained sexual maturity and were paired on Day 14, gentle aeration was added to each test chamber, and dissolved oxygen was measured daily until the end of the test in one replicate test chamber of each treatment and control group.

Duplicate water samples were collected from one test chamber of each treatment and control group two and one day(s) prior to the start of the test after conditioning the diluter for one to two days. Duplicate water samples also were collected from alternating replicate test chambers in each treatment and control group at the beginning of the test, approximately weekly during the test and at test termination to measure concentrations of the test substance. Additional samples were collected on Day 16 to confirm concentrations two days after gentle aeration was added to the test chambers at pairing.

Statistics: Test endpoints analyzed statistically for first-generation mysids were survival, reproduction (the number of live young produced per reproductive day), and growth (total body length and dry weight). The results of the statistical analyses were used to aid in the determination of the NOEC, LOEC and MATC. However, scientific judgment was used to determine if statistical differences were biologically meaningful, and if the data followed a concentration-dependent response. The NOEC was defined as the highest test concentration that produced no significant treatment-related effects on survival, reproduction or growth. The LOEC was defined as the lowest test concentration that produced a significant treatment-related effect on survival, reproduction or growth. The MATC was calculated as the geometric mean of the NOEC and LOEC.

Data from the negative and solvent control groups for each parameter were compared using an appropriate statistical test. Since no differences were detected between the two control groups ($p > 0.05$) for adult survival, reproduction and growth measurements, the control data were pooled for comparison among the treatment groups for those parameters. Differences were detected between the two control groups for juvenile mysid survival. Therefore, the treatment groups were compared to the negative control for the juvenile mysid survival endpoint.

Survival data was considered to be discrete-variable data, while reproduction and growth data were considered continuous-variable data. Discrete-variable data were analyzed using Chi-square and Fisher's Exact tests to identify treatment groups that showed a statistically significant difference from the pooled control for adult mysid survival and the negative control for juvenile mysid survival ($p \leq 0.05$). All continuous-variable data were evaluated for normality using the Shapiro-Wilk's test and for homogeneity of variance using Levene's test ($p = 0.01$). The data

for all parameters passed the assumptions of normality and homogeneity of variance. Those treatment means that were significantly different from the pooled control means were identified using Dunnett's test ($p \leq 0.05$). All statistical tests were performed using a personal computer with SAS software.

Findings:

Analytical data: Overall mean measured concentrations in test media were 84 - 98 % of nominal.

Table 137: Concentrations measured in exposure solution during the early life-stage exposure of mysi shrimps to S-2200 technical grade

S-2200 [µg ai/L] (nominal)	Measured concentration [mg ai/L]							Percent of nominal [%]
	Day 0			Day 36			Mean	
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200		
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.	n.a.
6.3	1.29	2.13	3.42	2.37	3.14	5.50	5.6	89
13	5.98	5.73	11.7	5.47	5.43	10.9	11	85
25	12.3	11.9	24.2	11.4	12.5	23.9	24	96
50	28.3	26.2	54.5	23.7	21.5	45.1	49	98
100	47.9	27.1	75.0	39.0	40.8	79.8	84	84

n.a…not applicable, LOQ…Limit of quantification (4 µg ai/L)

Survival and reproduction (juvenile, G_1 generation): Information on the juvenile survival to pairing (days 0-14) and the adult (G_1) survival (after pairing, days 15 – 36) see Table 138.

Table 138: Survival and Reproduction of saltwater mysids exposed to S-2200

S-2200 [µg ai/L] (mean measured)	% Juvenile Survival to Pairing (Day 15)	% Adult Survival to Test Termination (Day 36)	Mean Number of Young Producted per Reprodcuted Day ± SD	% of Female Producing Young [c]	Averg. Number of Young per Female [c]
Control	98.3	91.1	0.398 ± 0.192	88.9	7.6
Solvent control	84.7	81.0	0.491 ± 0.157	100	9.5
Pooled control	- [a]	86.2	0.445 ± 0.170	94.6	8.5
5.6	98.3	81.1	0.457 ± 0.236	94.1	9.2
11	91.7	72.9 **[b]	0.481 ± 0.152	88.2	8.8
24	88.3 *[b]	77.3	0.238 ± 0.106	63.2	4.2
49	100	74.5	0.357 ± 0.196	80.0	7.1
84	100	66.7 **	0.197 ± 0.163	57.9	3.9
NOEC (survival) = 49 µg ai/L NOEC (reproduction) = 84 µg ai/L					

* Statistically significant decrease in survival in comparison to the negative control using Fisher's Exact test ($p \leq 0.05$).
** Statistically significant decrease in survival in comparison to the pooled control using Fisher's Exact test ($p \leq 0.05$).
[a] There was a statistically significant difference in juvenile survival between the negative and solvent control groups ($p \leq 0.05$). Therefore, comparisons for juvenile survival were made to the negative control.

[b] While the decrease in survival was statistically significant in comparison to the negative/pooled control, it was not considered to be treatment-related since the difference was slight and was not dose-responsive.
[c] Statistical analysis were not performed on the percent of females producing young or the number of young per female.

Survival (G_2 generation): No assessment of the survival of second-generation mysids was conducted. The negative control, solvent control, 5.6, 24 and 49 µg ai/L treatment group mysid pairs produced the minimum number of live G_2 mysids required for a survival test (n=5). However, the number of available organisms was too small to draw conclusions relating to G_2 survival following G_1 mysid exposure to S-2200. The paucity of live offspring was not limited to the treatment groups. G_1 mysids in the control groups produced a total of 316 offspring but only 45 were alive. Biological observations of mortality and sub-lethal effects of these second generation juvenile mysids are presented in Table 139.

Table 139: Number of young G_2 saltwater mysids observed and enumerated per G_1 female each day (days 15 – 36)

S-2200 [µg ai/L] (mean measured)	**Number of G_2 juveniles (Day 36)**		
	Total	**Alive**	**Dead**
Control	136	11	125
Solvent control	168	34	134
5.6	157	81	76
11	-	-	-
24	80	6	74
49	141	9	132
84	-	-	-

Four replicates per treatment and control groups, five compartments (each contained 1 male and 1 female) per replicate

Growth: See Table 140.

Table 140: Growth of saltwater mysids exposed to S-2200

S-2200 [µg ai/L] (mean measured)	**Mean total length ± SD [mm]**		**Mean dry weight ± SD [mg]**	
	Males	**Females**	**Males**	**Females**
Control	8.54 ± 0.054	8.41 ± 0.297	0.977 ± 0.058	1.12 ± 0.115
Solvent control	8.50 ± 0.153	8.67 ± 0.255	0.991 ± 0.068	1.22 ± 0.125
Pooled control	8.52 ± 0.109	8.54 ± 0.292	0.984 ± 0.059	1.17 ± 0.125
5.6	8.26 ± 0.392	8.83 ± 0.089	0.845 ±0.151 *[a]	1.20 ± 0.075
11	8.59 ± 0.147	8.79 ± 0.217	0.996 ± 0.082	1.23 ± 0.218
24	8.40 ± 0.182	8.76 ± 0.150	0.982 ± 0.062	1.25 ± 0.078
49	8.59 ± 0.143	8.91 ± 0.166	0.960 ± 0.061	1.30 ± 0.093
84	8.04 ± 0.414 *	8.50 ± 0.039	0.944 ± 0.070	1.24 ± 0.172
NOEC (growth) = 49 µg ai/L				

* Statistically significant decrease in comparison to the pooled control using Dunnett's test ($p \leq 0.05$).
[a] While the decrease in weight was statistically significant in comparison to the pooled control, it was not considered to be treatment-related since the difference was slight and was not dose-responsive.

Conclusion: 34 d NOEC = 49 µg ai/L (growth, survival)

34 d LOEC = 84 µg ai/L
based on mean measured concentrations

Comment RMS: The RMS agrees on the validity of the study. However, considering the interpretation of the results of the study the RMS does not agree with the notifier. Considering the results on the adult (G_1) survival and the reproductive parameters no clear dose-response can be shown. Taking into account the results on the adult survival statistically significant effects were observed at the test concentrations 11 µg ai/L and 84 µg ai/L. The notifier argued that the effects at 11 µg ai/L are not treatment related since the decrease was slight and was not dose responsive. The RMS does not agree on this statement. Even though the effects at the test concentrations 24 and 49 µg ai/L were not considered statistically significant the RMS is of the opinion that the effects should be considered. Based on the dose-response curve it is not possible to identify a clear outlier as the percentage of adult survival within the treatment groups is close.
Hence, the RMS is of the opinion the NOEC (adult survival) should be 5.6 µg ai/L.
In addition, it should be considered that no assessment of the G_2 juvenile survival could be conducted as the mortalities in the treatment and control groups were too high.

Reference:	**S-2200 – Toxicity Test with Sediment-Dwelling Midges (*Chironomus riparius*) Under Static Conditions, Following OECD Guideline 219**
Author(s), year:	Picard, Christian R., 2012
Report/Doc. number:	Report No. ROW-0047, Study No. 13048.6671
Guideline(s):	OECD Guideline 219
GLP:	Yes
Deviations:	The protocol stated that 20 midge larvae will be added to each test vessel. In this study, greater than 20 midge larvae were inadvertently added to one replicate of the control group. A total of 21 midge emerged from the replicate and a percent emergence of 100% was utilised for statistical analysis. Based on the high emergence for all replicates in the control group and all other test groups, this deviation did not have a negative impact on the results or interpretation of the study.
Validity:	Acceptable

Material and methods:

Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G [benzyl-^{14}C] S-2200, radiochemical purity 98.1 – 98.9%, batch: CFQ40467
Reference substance:	MCBX, purity: 96.9%, batch: CTS08015 2-COOH-S-2200, purity: 99.0%, batch: 317-001-47-1 5-COOH-S-2200, purity: 97.6%, batch: 262-005-10-1
Test species:	Midge (*Chironomus riparius*)
Number of organisms:	4 replicates for each treatment level and for the solvent and water controls, 20 midge larvae per replicate
Age:	First instar, 3 days old
Type of test,	Static water/sediment test system (spiked water exposure), 28 d

duration:	
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.68, 1.5, 3.3, 7.3 and 16 mg ai/L
Initial measured:	- (control and solvent control), 0.64, 1.4, 2.7, 4.8 and 8.1 mg ai/L
Solvent:	Acetone (CAS No. 67-64-1)
Test system:	
Water quality:	Laboratory well water, hardness: 56 mg/L as $CaCO_3$, alkalinity: 24 mg/L as $CaCO_3$, pH = 7.0, conductivity: 270 µmhos/cm
Sediment:	Artificial sediment according to OECD 218 and 219 (6.0% sphagnum peat, 20% kaolin clay, 74% fine sand), 1.8% organic carbon content, pH = 7.0, particle size distribution: 77% sand, 4% silt and 19% clay
Size of test vessels:	600 mL glass beakers, 1.5 cm sediment layer
No. of replicates:	4 replicates per treatment level, control and solvent control for biological evaluation and 4 replicates per treatment level, control and solvent control for chemical analysis.
Test condition:	
Temperature:	19 - 20 °C
pH:	5.8 – 8.3
Hardness:	48 - 64 mg $CaCO_3$/L
Alkalinity:	16 – 46 mg $CaCO_3$/L
Conductivity:	490 – 630 µmhos/cm
Ammonia:	0.15 – 0.60 mg N/L
O_2 content:	6.7 – 9.1 mg O_2/L (> 60 % saturation)
Light regime:	16 hours light / 8 hours darkness, intensity: 520 – 790 lux
Feeding:	The midge larvae were fed a suspension of fish food, initially at a daily rate of about 1 mL per vessel until day 10 and thereafter at approximately 2.0 mL per vessel and day until the end of the test.
Test parameters:	Midges (behaviour, emergence,…) were examined at test day -1 and daily thereafter, until test termination (day 28). The sex, the time point of emergence and the number of emerged midges were recorded daily from day 10 post-treatment onwards. For chemical analysis (HPLC-UV/RAM) of S-2200 in the overlaying water column, pore water and sediment samples were taken from fresh at day 0 (test initiation), day 7 and day 28. Measurements of temperature, pH and dissolved oxygen concentration were made at daily intervals in an alternate replicate vessel of each treatment level and the controls during the 28-day exposure. In addition, measurement of temperature, pH and dissolved oxygen were made on the day the test organisms were added (day -1) and application of the test substance (day 0) and at test termination (day 28) in each exposure vessel. Total hardness, alkalinity, conductivity, and total ammonia of the test solution were determined on day 0 and at test termination in a composite sample from the highest treatment level and control solution.
Statistics:	Comparison of control groups: t-test for homogeneous variances. Data for all endpoints (midge emergence, male/female combined development rate): Normal distribution and homogeneity of variance: Shapiro Wilks' Test and Bartlett's Test. Emergence and development rate: Equal Variance t Two-Sample Test EC_{50}, NOEC: empirically estimated to be greater than the highest

concentration tested.

Findings:

Analytical data: See Table 141

Table 141: Measured concentrations of S-2200 in the overlaying water, the pore water and the sediment

S-2200 [mg ai/L]	Day 0		Day 7		Day 28	
	Measured concentration	% of nominal	Measured concentration	% of nominal	Measured concentration	% of nominal
	Overlaying water [mg total ^{14}C-residues/L]					
Control	< 0.0050	n.a.	< 0.0051	n.a.	< 0.0050	n.a.
Solvent control	< 0.0050	n.a.	< 0.0050	n.a.	< 0.0050	n.a.
0.68	0.64	94	0.31	45	0.26	39
1.5	1.4	94	0.76	51	0.60	40
3.3	2.7	80	1.6	49	1.3	41
7.3	4.8	66	3.8	52	3.1	42
16	8.1	51	5.6	35	5.4	34
	Pore water [mg total ^{14}C-residues/L]					
Control	< 0.012	n.a.	< 0.012	n.a.	< 0.012	n.a.
Solvent control	< 0.012	n.a.	< 0.012	n.a.	< 0.012	n.a.
0.68	0.014	n.a.	0.11	n.a.	0.21	n.a.
1.5	0.095	n.a.	0.22	n.a.	0.51	n.a.
3.3	0.12	n.a.	0.46	n.a.	1.2	n.a.
7.3	0.36	n.a.	1.2	n.a.	2.8	n.a.
16	1.2	n.a.	1.7	n.a.	4.2	n.a.
	Sediment [mg total ^{14}C-residues/kg]					
Control	< 0.0053	n.a.	< 0.0052	n.a.	< 0.0053	n.a.
Solvent control	< 0.0053	n.a.	< 0.052	n.a.	< 0.054	n.a.
0.68	< 0.0053	n.a.	0.69	n.a.	0.62	n.a.
1.5	< 0.12	n.a.	1.6	n.a.	1.2	n.a.
3.3	1.1	n.a.	2.9	n.a.	3.7	n.a.
7.3	4.4	n.a.	9.5	n.a.	8.5	n.a.
16	10	n.a.	13	n.a.	13	n.a.

n.a…not applicable

Effects: Sex ratio: No relationship between treatment and sex ratio was found; therefore number of males and females midges was pooled for further endpoint calculations.
Development rate, midge emergence: No significant differences (Bonferroni's Adjusted t-Test) in mean percent emergence and in combined male/female midge development rate were identified.
For further details see Table 122

Table 142: Effects of S-2200 on midge (*C. riparius*) in a water-spiked test

S-2200 [mg ai/L] Mean measured (overlying water)	Number of emerged midges (out of 80)	Emergence of larvae			Mean development rate [1/d] [a]
		total [%]	male [%]	female [%]	
Control	75	93	52	48	0.0720

S-2200 [mg ai/L] Mean measured (overlying water)	Number of emerged midges (out of 80)	Emergence of larvae			Mean development rate [1/d] [a]
		total [%]	male [%]	female [%]	
Solvent control	77	96	45	55	0.0720
Pooled control	152	95	49	51	0.0720
0.64	74	93	50	50	0.0754
1.4	76	95	43	57	0.0747
2.7	76	95	53	47	0.0773
4.8	79	99	59	41	0.0767
8.1	73	91	52	48	0.0719
NOEC		8.1 mg/L			8.1 mg/L
LOEC		> 8.1 mg/L			> 8.1 mg/L
EC_{50}		> 8.1 mg/L			> 8.1 mg/L

[a] Mean developmental rate is based on combined sex data.

Conclusions: NOEC = 8.1 mg ai/L (total emergence of larvae, mean development rate)
LOEC > 8.1 mg ai/L
EC_{50} > 8.1 mg ai/L
based on mean measured concentrations

Reference: **S-2200: A life cycle toxicity test with the freshwater amphipod (*Hyalella azteca*) using spiked sediment**
Author(s), year: Thomas, Susan T., Martin, Kathy H. and Gallagher, Sean P., 2013c
Report/Doc. number: Report No. ROW-0078, Study No. VP-38509
Guideline(s): U.S. EPA OPPTS 850.1770 (draft, 1996), US EPA 600/R-99/064
GLP: Yes
Deviations: Organisms from the culture were collected to be measured for dry weight on Day 0 instead of prior to test initiation as stated in the protocol. Additionally only 20 organisms were used for the determination of dry weight at the start of the test instead of at least 40 as described in the protocol.
It was the Study Director's decision to collect organisms from the culture on Day 0 instead of prior to test initiation. Organisms collected prior to test initiation would not have been as representative in age or size of the organisms used in the test as those collected at the same time the test was being imitated. Additionally, the biologist inadvertently used 20 organisms instead of at least 40. Since a weight was still obtained to represent the weight of the organisms at the start of the test, and the organisms used were the same age and size as those used in the test, these deviations from the protocol had no adverse impact upon the results or interpretation of the study.
The organisms were not fed 2 mg of wheat grass per test compartment on Days 0, 1 and 2 of the test.
Reason: Biologist oversight. The biologist inadvertently did not feed wheat grass to the organisms for the first two days of the test. Once the error was discovered, wheat grass was added to the feeding regime. Since the organisms in the test had measureable growth and reproduction, this

	oversight did not have any adverse impact on the results or interpretation of the study. pH was not measured in the pore water at the beginning, approximate middle and end of the test. Reason: Biologist oversight. The pH of the pore water was inadvertently not collected during the test. The pH in the overlying water was consistently 8.0 – 8.2 throughout the test and the pH in the sediment ranged from 6.9 to 7.7 during the test. This deviation from the protocol is not believed to have had an adverse impact on the results or interpretation of the study. On Days 28, 35 and 42 of the test an assessment of survival was made for each treatment and control replicate; however, general observations were inadvertently not made. Reason: Biologist oversight. This deviation from the protocol had no adverse impact upon the results or interpretation of the study. On Day 35 of the test, after counting, the surviving adults were returned to the test system in clean, labeled, water-only test compartments instead of being returned to the test compartments from which they were removed. Reason: This change in procedure is believed to be better for organism health and for better water quality. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.
Validity:	Acceptable

Material and methods:	
Test substance:	S-2200 technical grade, purity: 93.4 %, batch: ST-0811G
Reference substances:	S-2354 (S-2200 *S*-isomer), purity: 99.7 %, batch: 060020653 S-2167 (S-2200 *R*-isomer), purity: 100 %, batch: 060020652
Test species:	Freshwater amphipod (*Hyalella azteca*)
Number of organisms:	12 replicates each with 10 amphipods per treatment, control and solvent control. 1 additional replicate per treatment and controls for measurments of the water and sediment quality. 3 additional replicate per treatment and controls for analytical measurements.
Age:	Juvenile amphipods, 7 days old
Type of test, duration:	Flow-through test, 42 days
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.3, 1.0, 3.0, 10 and 30 mg ai/kg sediment
Solvent:	Acetone (CAS No. 67-64-1)
Test conditions:	
Water quality:	Well water, total hardness of 134 mg/L as $CaCO_3$, total alkalinity of 177 mg/L as $CaCO_3$, specific conductivity of 369 - 486 µS/cm, Ammonia of ≤ 0.17 – 0.705 mg/L as NH_3
Temperature:	22 ± 1 °C
pH:	8.0 – 8.2 (overlying water), 6.9 – 7.7 (sediment)
O_2 content:	7.0 – 9.0 mg O_2/L (> 60 % saturation)

Sediment quality:	Artificial sediment (according Kemble et al., is similar to the OECD guideline 218, but uses alpha-cellulose as its source of organic matter instead of peat moss), 3% organic carbon, pH = 6.5
Particle size distribution:	80% industrial quartz sand, 14% silt and clay (kaolin clay), 5% alpha-cellulose and 1% humic acid and dolomite
Light regime:	16 hours light / 8 hours darkness (516 lux)
Feeding:	During the exposure, the freshwater amphipods were fed 2 mg of wheat grass and TetraMin® flake food once daily. Organisms were not fed on the last day of the test, nor were they fed wheat grass on days 0, 1 and 2 of the test.
Test parameters:	Exposure of the organisms was conducted over a 42-day period, including a 28-day sediment/overlying water exposure and a 14-day water only exposure for monitoring reproduction. During the exposure period, assessments of survival and general observations of individuals exhibiting signs of toxicity or abnormal behavior were made daily. Adult survival was evaluated on Days 28, 35 and 42. Growth was evaluated on Days 28 and 42 and reproduction was evaluated on Days 28, 35 and 42. On Day 0, a subset of 20 organisms from the culture was placed in an oven for the determination of dry weight. On Day 28 of the test, amphipods were removed from the sediment by sieving using a 0.425 mm sieve and a shallow sorting pan to assess survival. Immobile organisms isolated from the sediment or overlying water were considered dead. Four of the replicate compartments from each treatment and control group were removed from the test for growth determination. The surviving amphipods from these four replicates were preserved in vials containing an 8% sugar formalin solution for later measurement of length and dry weight. The surviving amphipods from the remaining eight replicates were transferred to clean corresponding water-only beakers for the remainder of the test. On Day 35 and at the end of the test, the survival of adult amphipods was determined by removing all the adults and young from each compartment and counting them. The gender of the adults was determined. On Day 35 after counting, the surviving adults but not the young, were transferred to clean water-only test compartments. On Day 42, the surviving adults (not the young) were preserved in an 8% sugar formalin solution for growth assessments. Any young observed on Day 28 were noted in the raw data. After the in-life of the test had terminated, the body length of each of the preserved organisms was determined by measuring from the base of the first antennae to the tip of the third uropod along the curve of the dorsum to the nearest 0.01 mm. First the organisms were arranged on a slide and photographs were taken. ImagePro photo software was used to measure the organisms. After the photos were taken, the organisms were transferred to tared weigh pans by replicate and the organisms and pans were dried in an oven to determine the dry weight of the organisms. Prior to Day 0, shortly after preparation, samples were collected from the 0.03, 0.1, 0.3, 1.0 and 3.0 mg a.i./mL stock solutions. Sediment, pore water and overlying water samples were collected from the analytical replicates from each test concentration and control on Days 0, 14 and 28. Sediment, pore water and overlying water samples were processed for subsequent analysis on the day of the collection or stored refrigerated (water) or frozen (sediment) until analysis.

Statistics: The results of the test were based on nominal concentrations in the sediment. Statistical analyses were performed to evaluate the difference between the treatment groups and the control groups for adult survival, body length, dry weight and reproduction (number of young per surviving female). The percent survival data precluded the calculation of an LC_{50} as all concentrations tested had greater than 50% survival. The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival, growth and reproduction data.

All statistical analyses were performed using TOXSTAT version 3.5 or SAS version 8.2 (7, 8). The data from the negative and solvent control groups were compared using a t-test. There were no statistically significant differences between the negative and solvent control groups for survival, reproduction or growth, therefore the treatment groups were compared to the negative control replicates. There was a problem with transfer of test organisms to test vessels in the negative control, 1.0 and 10 mg a.i./kg treatment groups on Day 28, resulting in lower survival numbers in these groups on Day 29. The lower survival was not considered to be treatment related since mortality was evident in the control as well as the two treatment groups, and because of a lack of a dose response relationship as evident by 100% survival in the highest treatment group. Therefore, statistical analysis on 42-day survival, length, weight and reproduction were performed by only comparing the solvent control to the 0.30, 3.0 and 30 mg a.i./kg treatment groups.

Survival data was evaluated for normality (Chi-Square) and homogeneity of variances (Bartlett's). Since the Day 28 survival data was not found to be normal or homogenous, transformations of the data were tried. Transforming the data did not correct the problem; therefore, a non-parametric procedure (Kruskal-Wallis) was used to identify statistically significant differences between the treatment groups and the negative control. The Day 42 survival data was found to be normal (Chi Square) and homogeneous (Levene's); therefore, the treatment groups were compared to the solvent control using a Bonferroni t-test as well as a Kruskal-Wallis test.

Growth was evaluated by looking at both length and weight data. The Day 28 and Day 42 length and weight data were evaluated for normality (Shapiro-Wilk) and homogeneity of variences (Levene's). After the data were deemed normal with homogenous variance, the data were analyzed using Dunnett's test to identify the treatment groups that were different from the negative control for the Day 28 data or from the solvent control for Day 42 data.

The reproduction data were evaluated for normality (Anderson-Darling) and homogeneity of variances (Levene's). After the data was deemed normal with homogeneous variance, the data was analyzed using Dunnett's test to identify those treatment levels that were statistically different ($p < 0.05$) from the solvent control group.

Findings:

Analytical data: The results of the test were based on nominal concentrations in the sediment.

Table 143: Concentrations measured in exposure solution during the life cycle test of freshwater amphipod to S-2200 technical grade

S-2200 [µg ai/L] (nominal)	Measured concentration						
	Day 0			Day 28			Mean measured
	S-2167	S-2354	S-2200	S-2167	S-2354	S-2200	
Sediment [mg ai/kg]							
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
0.3	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
1.0	0.281	0.356	0.637	0.187	0.248	0.435	0.50
3.0	0.210	0.789	0.999	0.438	0.449	0.887	1.1
10	3.57	3.45	7.02	1.63	1.63	3.26	5.0
30	9.91	9.92	19.8	4.51	4.47	8.98	14.0
Overlaying water [mg ai/L]							
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
0.3	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
1.0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
3.0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
10	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
30	0.088	0.106	0.195	< LOQ	< LOQ	< LOQ	n.a.
Pore water [mg ai/L]							
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
0.3	0.0877	0.140	0.228	0.0641	0.0622	0.126	0.181
1.0	0.347	0.406	0.753	0.179	0.213	0.392	0.582
3.0	1.19	1.27	2.46	0.561	0.618	1.18	1.83
10	4.21	4.35	8.56	2.04	2.05	4.09	6.38
30	12.8	13.05	25.8	6.20	6.49	12.7	19.1

n.a…not applicable, LOQ…Limit of quantification (0.1 mg ai/kg in the sediment and 0.1 mg ai/L in the overlying water)

Biological effects: Effects on the survival and growth parameter (weight and length) see Table and.

Behavioural effects (climbing the walls of the test compartment, swimming in the water column, trapped in the air/water interface, observed on the surface of the sediment) were observed at all test concentrations including the control groups.

Table 144: Effects (survival, growth) on freshwater amphipods (*Hyalella azteca*) exposed to technical S-2200 at day 28

S.2200 [mg ai/kg] (nominal)	Mean percent survival ± SD [%]	Average body length ± SD [mm]	Average individual dry weight ± SD [mg]
Control	94.2 ± 7.9	4.98 ± 0.16	0.73 ± 0.066
Solvent control	95.8 ± 7.9	4.77 ± 0.18	0.73 ± 0.077
0.3	91.7 ± 10	4.75 ± 0.15	0.71 ± 0.048
1.0	99.2 ± 2.9	4.89 ± 0.17	0.78 ± 0.045
3.0	94.2 ± 6.7	4.88 ± 0.12	0.79 ± 0.064
10	95.8 ± 6.7	4.73 ± 0.30	0.70 ± 0.117
30	96.7 ± 4.9	4.64 ± 0.22 *	0.72 ± 0.071

SD…Standard deviation
* Statistically significant compared to the negative control using Dunnett's test ($p \leq 0.05$)

Table 145: Effects (survival, growth) on freshwater amphipods (*Hyalella azteca*) exposed to technical S-2200 at day 42

S.2200 [mg ai/kg] (nominal)	Mean percent survival ± SD [%] Day 28 to 42 [a]	Average body length ± SD [mm]	Average individual dry weight ± SD [mg] [a]	Average number of young/surviving female ± SD [a]
Control	79.9 ± 25 [b]	5.85 ± 0.18	0.90 ± 0.061	10.7 ± 4.8
Solvent control	97.2 ± 5.3	5.65 ± 0.31	0.89 ± 0.050	13.8 ± 3.7
0.3	93.1 ± 10.9	5.56 ± 0.19	0.87 ± 0.060	11.0 ± 4.2
1.0	27.5 ± 32 [b]	5.44 ± 0.32	0.94 ± 0.127	16.6 ± 5.1
3.0	94.7 ± 5.7	5.73 ± 0.34	0.96 ± 0.093	15.1 ± 7.6
10	38.8 ± 16 [b]	5.23 ± 0.42	0.74 ± 0.148	7.5 ± 10.1
30	100 ± 0	5.44 ± 0.28	0.83 ± 0.090	10.1 ± 4.5

SD…Standard deviation
[a] Average survival, length and weight of surviving organisms in eight replicates terminated on Day 42. Since there was a problem with transfers in the negative control, 1.0 and 10 mg a.i./kg treatment groups on Day 28, resulting in low survival numbers in these groups on Day 29, statistical analysis on survival, length, weight , and reproduction were performed by comparing the solvent control to the 0.30, 3.0 and 30 mg a.i./kg treatment groups.
[b] The lower values in the negative control, 1.0 and 10 mg ai/kg treatment groups were attributed to injury during transfer that resulted in 14%, 58%, and 43% mortality in those groups on Day 29, less than 24 hours after transfer from beakers with water and treated sediment to untreated dilution water only. The lower survival numbers in these groups were not considered to be treatment related since mortality was evident in the control as well as two treatment groups, and because of a lack of a dose response relationship as evident by 100% survival in the highest treatment group.

Conclusion: 42 d NOEC = 10 mg ai/kg (growth)
42 d LC_{50} > 30 mg ai/kg
42 d EC_{50} > 30 mg a.s./kg (reproduction)
42 d LOEC = 30 mg a.s./kg (growth)
based on nominal concentrations

Validity criteria: All validity criteria were met for the study. The average survival of *Hyalella azteca* on Day 28 was > 80% in the negative and solvent control groups. The average length of *Hyalella azteca* on Day 28 was > 3.2 mm in the negative and solvent control groups and reproduction by Day 42 was > 2 young per female in the negative and solvent control groups. There is a new criteria being discussed that recommends an 8 x increase from initial weight at test initiation to 28 days and a 10 x increase by 42 days. At the beginning of the test, a group of 20

organisms from the culture was impartially selected and used for the determination of dry weight. It was determined that the average individual dry weight of those twenty organisms was 0.020 mg. This represents a 37 fold increase in average weight in the negative control by Day 28 of the test and a 45-fold increase by Day 42.

Comment RMS: The study is considered valid taking into account the validity criteria given in the test guideline.

Considering the adverse effects on survival at Day 42 the notifier argued that, the results on survival (42 d) should not be included in the assessment. The lower values in the negative control, 1.0 and 10.0 treatment groups were attributed to injury during transfer that resulted in 14%, 58%, and 43% mortality in those groups on Day 29, less than 24 hours after transfer from beakers with water and treated sediment to untreated dilution water only. The lower survival numbers in these groups were not considered to be treatment related since mortality was evident in the control as well as the two treatment groups, and because of a lack of a dose response relationship as evident by 100% survival in the highest treatment group. The RMS agrees on the statement provided by the notifier, an evaluation of the survival of freshwater amphipods after 42 days is not possible on the available information.

The results of the study are based on nominal test concentrations in the sediment. However, the mean measured concentrations in the sediment are < 80% of the nominal test concentrations. Hence, the RMS is of the opinion that the results should be based on mean measured concentrations. Under consideration of mean measured concentrations the 28 d NOEC based on growth was determined to be 5.0 mg ai/kg sediment.

In conclusion, the RMS agrees on the NOEC based on mean measured concentrations. However, there are some uncertainties regarding the statistical analysis of reproduction considering the high mortality observed in the negative control, 1.0 and 10.0 treatment groups.

Reference:	**S-2200: A life cycle toxicity test with the marine amphipod (*Leptocheirus plumulosus*) using spiked sediment**
Author(s), year:	Thomas, Susan T., Martin, Kathy H. and Gallagher, Sean P., 2013a
Report/Doc. number:	Report No. ROW-0073, Study No. VP-38066
Guideline(s):	US EPA 600/R-01/020
GLP:	Yes
Deviations:	The target test temperature was maintained at 25 ± 2°C instead of 25 ± 1°C. The reason for this deviation was that the environmental chamber used for the test was able to maintain temperature within ± 2°C of the target temperature. All organisms in the controls appeared healthy throughout the test. This deviation from the protocol had no adverse impact upon the results or interpretation of the study. A single subset of 20 organisms from the culture was used to determine the initial neonate dry weight instead of three subsets of 20 organisms as descried in the protocol. The reason for this deviation was Biologist oversight. The 20 organisms measured provided a representative subsample of the test organisms. This slight deviation from the protocol had no adverse impact upon the results or interpretation of the study. The procedure to determine the initial neonate dry weight was performed on Day 0, immediately following test initiation instead of prior to test initiation as prescribed in the protocol. The reason for this deviation was that the neonates to be used in the test were received form Chesapeake Cultures on the day the test was to be initiated and the organisms were used immediately upon receipt. Obtaining a weight of the organisms prior to the start of the test was not possible. Measurement of initial neonate dry weight immediately following test initiation provided an accurate representation of the weight of the organisms being used in the test. This deviation from the protocol had no adverse impact upon the results or interpretation of the study. The measurement of pH in the pore water was not obtained. The reason for this deviation was Biologist oversight. Measurement of pH in overlying water and sediment provides pH conditions in the test system. This deviation from the protocol had no adverse impact upon the results or interpretation of the study. Documentation of the sediment surface appearance was not maintained daily during the test. The reason for this deviation was Biologist oversight. The sediment surface was observed daily while biological observations were being performed, but appearance of the sediment surface was not documented. However, biologists regularly record any unusual observations of changes in the test system (e.g. fungal growth) if they appear. This slight deviation from the protocol had no adverse impact upon the results or interpretation of the study.
Validity:	Acceptable

Material and methods:

Test substance:	S-2200 technical grade, purity: 93.4 %, batch: ST-0811G
Reference substances:	S-2354 (S-2200 *S*-isomer), purity: 99.4 %, batch: AS 2263a S-2167 (S-2200 *R*-isomer), purity: 99.8 %, batch: AS 2262a
Test species:	Marine amphipod (*Leptocheirus plumulosus*)

Number of organisms:	Five replicate test chambers for each treatment and control groups, with 20 amphipods in each test chamber, for a total of 100 amphipods per test concentration. Three additional replicates were added to each treatment and control group for water quality measurements and measurements of sediment pH. Three additional replicates were added in each treatment and control group for analytical sampling of water and sediment.
Age:	Neonates, size: 2 – 4 mm
Type of test, duration:	Static-renewal test, 28 days
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.3, 1.0, 3.0, 10 and 30 mg ai/kg
Solvent:	Acetone (CAS No. 67-64-1)
Test conditions:	
Overlaying water:	Sand-filtered natural seawater, salinity: 20 ‰, pH = 7.8 – 7.9
Temperature:	25 ± 2 °C
pH:	7.7 – 8.9 (overlying water), 6.6 – 7.7 (sediment)
O_2 content:	6.1 – 7.4 mg O_2/L (> 60 % saturation)
Ammonium	< 0.17 mg/L as NH_3 (overlying water), 0.414 – 2.52 mg/L as NH_3 (pore water)
Sediment quality:	Natural marine sediment, organic carbon: 1.6 %, pH 7.6
Particle size distribution:	43% sand, 46% silt and 11% clay
Light regime:	16 hours light / 8 hours darkness (518 lux at the surface of the water)
Feeding:	During the test the organisms were fed three times per week (after the renewal of the overlying water) TetraMin® flake food. Between days 0 and 13, 20 mg of flake food were added to each test chamber. Between days 14 and 28, 40 mg of flake food were added to each test chamber.
Test parameters:	On Day 0, a subset of 20 organisms from the culture was placed in an oven for the determination of dry weight. The test chambers were observed daily during the test to determine the number of mortalities and the number of individuals exhibiting signs of toxicity or abnormal behavior. On Day 28 of the test, amphipods were removed from the sediment by sieving between a 0.5 mm (adults) and a 0.25 mm (young) sieve. The numbers of live or dead amphipods were enumerated as well as the number of offspring. Missing animals were recorded as dead. The surviving organisms were grouped by replicate for each treatment group and dried in an oven to determine the average individual dry weight. The target test temperature during the study was 25 ± 1°C. Temperature was measured in the overlying water of one alternating replicate test chamber of each experimental group daily during the test using a hand-held liquid-in-glass thermometer. Temperature was also monitored continuously in a beaker of water placed adjacent to the test chambers using a Min/Max thermometer. The Min/Max thermometer was verified with a hand-held NIST thermometer prior to test initiation. Dissolved oxygen was measured in the overlying water from one alternating replicate test chamber of each experimental group daily during the test. Measurements of pH in the overlying water were made in one alternating replicate from each experimental group at the beginning and end of the test and three times per week during the test. The pH of the

sediment was also measured in a sacrificial water quality replicates for each treatment group and control on Days 0, 14 and 28 of the test. Ammonia measurements in the overlying water and pore water were made from one of the sacrificial water quality replicates for each treatment and control group on Days 0, 14 and 28 of the test. Salinity measurements were made in a sample of pre-renewal overlying water in one alternating replicate from each treatment group and control at the beginning and end of the test and approximately three times per week during the test.

Prior to Day 0, shortly after preparation, samples were collected from the 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 mg a.i./mL stock solutions. Sediment, pore water and overlying water samples were collected from the analytical replicates from each test concentration and control on Days 0, 14 and 28. Sediment, pore water and overlying water samples were processed for subsequent analysis on the day of the collection or stored refrigerated (water) or frozen (sediment) until analysis.

Statistics: The results of the test were based on nominal concentrations in the sediment. The percent survival data precluded the calculation of an LC_{50} as all concentrations tested had greater than 50% survival. The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival, growth and reproduction data.

The growth rate was determined as the individual weight of the organisms by day and was calculated using the following formula: (mean individual adult dry weight – mean individual neonate dry weight)/28 days. Reproduction is reported as the number of young per surviving adult in each replicate.

All statistical analyses were performed using TOXSTAT version 3.5. The data from the negative and solvent control groups were compared using a t-test. There were no statistically significant differences between the negative and solvent control groups for survival, reproduction or growth, therefore the treatment groups were compared to the negative control replicates. Survival, growth rate and reproduction data were evaluated for normality (Chi-Square) and homogeneity of variances (Levene's test for survival and Bartlett's test for growth and reproduction). After the data was deemed normal with homogeneous variance, the data was analyzed using a Dunnett's test to identify those treatment levels that were statistically different ($p < 0.05$) from the negative control group.

Findings:

Analytical data: The results of the test were based on nominal concentrations in the sediment.

Effects: See Table 126

Table 146: Concentrations measured in exposure solution during the life cycle test of freshwater amphipod to S-2200 technical grade

S-2200 [µg ai/L] (nominal)	Sum of measured concentrations			Mean measured [a]
	Day 0	Day 14	Day 28	
Sediment [mg ai/kg]				
Control	< LOQ	< LOQ	< LOQ	n.a.
Solvent control	< LOQ	0.198 [b]	0.959 [b]	n.a.
0.3	< LOQ	0.277	0.532	0.270
1.0	< LOQ	< LOQ	0.437	0.146
3.0	1.12	0.754	0.618	0.831
10	5.73	4.52	4.20	4.82
30	12.8	11.4	6.79	10.3
Overlaying water [mg ai/L]				
Control	< LOQ	< LOQ	< LOQ	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	n.a.
0.3	< LOQ	< LOQ	< LOQ	n.a.
1.0	< LOQ	< LOQ	< LOQ	n.a.
3.0	< LOQ	0.021	< LOQ	n.a.
10	0.053	0.073	0.048	n.a.
30	0.156	0.252	0.124	n.a.
Pore water [mg ai/L]				
Control	< LOQ	< LOQ	< LOQ	n.a.
Solvent control	< LOQ	< LOQ	< LOQ	n.a.
0.3	0.017	< LOQ	< LOQ	0.00567
1.0	0.057	0.027	0.022	0.0353
3.0	0.177	0.108	0.079	0.121
10	0.675	0.403	0.290	0.456
30	2.10	1.48	1.11	1.56

n.a…not applicable, LOQ…Limit of quantification
[a] Calculated as the sum of the matrix subtracted isomers divided by the soil content of the sample.
[b] The measured values in the solvent control may have been a result of matrix interference

Biological effects: See Table 127

Table 147: Effects (survival, growth, reproduction) on marine amphipods (*Leptocheirus plumulosus*) exposed to technical S-2200 at day 28

S.2200 [mg ai/kg] (nominal)	Mean percent survival ± SD [%]	Growth rate ± SD	Number of young/surviving female
Control	95 ± 11	0.014 ± 0.011	1.0 ± 0.79
Solvent control	97 ± 4.5	0.015 ± 0.011	0.44 ± 0.41
0.3	94 ± 5.5	0.029 ± 0.019	1.0 ± 1.5
1.0	87 ± 16	0.025 ± 0.020	1.0 ± 2.1
3.0	87 ± 26	0.0096 ± 0.0078	0.55 ± 0.82
10	97 ± 2.7	0.052 ± 0.012	3.9 ± 1.0
30	74 ± 18	0.045 ± 0.0069	0.37 ± 0.21

SD…Standard deviation

Conclusion: 28 d NOEC = 30 mg ai/kg
28 d LC_{50} > 30 mg ai/kg
28 d EC_{50} > 30 mg ai/kg
28 d LOEC > 30 mg ai/kg (growth, reproduction)
based on nominal concentrations

Comment RMS: The RMS agrees on the validity of the study based on the US EPA test guideline. However, the endpoints were expressed based on nominal concentrations even though the mean measured concentrations in the sediment were determined to be below 80% of the nominal test concentrations. Hence, the RMS is of the opinion that the NOEC should be 10.3 mg ai/kg sediment based on mean measured concentrations.

5.4.3 Algae and aquatic plants

Reference:	**S-2354 (*S*-Isomer of S-2200) – 72-Hour Toxicity Test with the Freshwater Green Alga, *Pseudokirchneriella subcapitata*, Following The Official Journal of the European Communities L383A, Method C.3**
Author(s), year:	Softcheck, Katherina A., 2012c
Report/Doc. number:	Report No. ROW-0051, Study No. 13048.6705
Guideline(s):	OECD Guideline 201, EC Guideline L383A - C.3
GLP:	Yes
Deviations:	None relevant
Validity:	Acceptable

Material and methods:

Test substance:	S-2354 (S-2200 *S*-isomer), purity: 99.7%, batch: 060020653
Test species:	Green alga (*Pseudokirchneriella subcapitata)*, class Chlorophyceae
Number of organisms:	1 x 10^4 cells/mL; 3 replicates per treatment group and medium control and 6 replicates per solvent control
Type of test, duration:	Static test, 72 hours
Applied concentrations:	
Nominal:	0 (medium and solvent control), 0.94, 1.9, 3.8, 7.5 and 15 mg/L
Measured (mean):	- (medium and solvent control), 0.79, 1.6, 3.5, 6.0 and 12 mg/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2), 0.1 mL/L
Test conditions:	
Test medium:	Algal Assay Procedure (AAP) medium (according to guideline), initial pH adjusted to 7.5 ± 0.1
Temperature:	24 - 25 °C
pH:	7.1 – 7.3 (0 h), 7.7 – 8.6 (72 h)
Conductivity:	68 - 89 µmhos/cm (0 h), 67 - 89 µmhos/cm (72 h)
Incubation:	Continuous illumination at 4500 to 5900 lux
Test parameters:	Cell counts were estimated using a haemocytometer and microscope. Observations of the health and morphology of the algal cells were made under the microscope on each study day. For chemical analysis (LC/MS/MS method) of test the substance, samples of test solution were taken at test initiation, after 72 h and at test termination. Measurements of pH and conductivity were made at initiation and at termination, light intensity was measured at daily intervals and temperature was monitored continuously.
Statistics:	Normal distribution and homogeneity of variance: Shapiro Wilks' Test and Bartlett's Test Significance of effects compared to the control: Bonferroni's adjusted t-Test

Findings:

Analytical data:	Mean measured concentrations were in the range of 80 - 91% of nominal concentrations over the whole test duration.
Morphological effects:	No effects on the morphology and appearance of the cells were observed during the study period.
Biomass, growth rate	See Table 128 and 129

and cell density:

Table 148: Cell density of *P. subcapitata* after 24, 48 and 72 h of exposure to S-2354

S-2354 [mg/L] (mean measured)	**Cell density (x 10^4 cells/mL), (± SD)**		
	24 h	**48 h**	**72 h**
0 (control)	8.00 (1.75)	40.75 (4.67)	138.33 (27.14)
0 (solvent control)	6.88 (1.81)	31.33 (5.52)	115.22 (21.91)
0.79	7.00 (1.25)	32.08 (14.54)	127.64 (23.23)
1.6	6.00 (0.66)	35.50 (7.47)	117.19 (20.41)
3.5	3.83 (0.72)	31.50 (5.27)	124.17 (27.25)
6.0	4.25 (0.90)	30.08 (5.91)	103.58 (14.75)
12	5.33 (2.13)	18.00 (2.22)	50.75 (5.06)

Table 149: Effects of technical S-2354 on the green alga *P. subcapitata*

S-2354 [mg/L] (mean measured)	**Percent inhibition relative to the solvent control [%]**	
	Biomass (0 – 72 h)	**Growth rate (0 – 72 h)**
0 (control)	-	-
0 (solvent control)	-	-
0.79	- 8	- 2
1.6	- 4	- 1
3.5	- 2	- 2
6.0	10	2
12	51 *	17 *
NOEC	6.0 mg/L	6.0 mg/L
EC_{10}	5.2 mg/L (n.a. – 8.3 mg/L)	8.8 mg/L (6.0 - 10 mg/L)
EC_{20}	6.9 mg/L (n.a. – 9.1 mg/L)	> 12 mg/L (n.a.)
EC_{50} (95 % C.l.)	12 mg/L (9.1 – n.a. mg/L)	> 12 mg/L (n.a.)

* Significantly different compared to the solvent control, based on Bonferroni's adjusted t-Test
Negative values indicate an increase of algal growth
n.a… not applicable, corresponding 95% confidence limit could not be calculated

Conclusion: 72 h E_bC_{50} = 12 mg/L
72 h E_rC_{50} = > 12 mg/L
72 h NOEC = 6.0 mg/L (biomass and growth rate)
based on mean measured concentrations.

Reference:	**S-2167 (*R*-Isomer of S-2200) – 72-Hour Toxicity Test with the Freshwater Green Alga, *Pseudokirchneriella subcapitata*, Following The Official Journal of the European Communities L383A, Method C.3**
Author(s), year:	Softcheck, Katherina A., 2012b
Report/Doc. number:	Report No. ROW-0050, Study No. 13048.6703
Guideline(s):	OECD Guideline 201, EC Guideline L383A - C.3
GLP:	Yes
Deviations:	None
Validity:	Acceptable

Material and methods:

Test substance:	S-2167 (S-2200 *R*-isomer), purity: 100%, batch: 060020652
Test species:	Green alga (*Pseudokirchneriella subcapitata)*, class Chlorophyceae
Number of organisms:	1×10^4 cells/mL; 3 replicates per treatment group and medium control and 6 replicates per solvent control
Type of test, duration:	Static test, 72 hours
Applied concentrations:	
Nominal:	0 (medium and solvent control), 0.078, 0.16, 0.31, 1.3, 2.5 and 5.0 mg/L
Measured (mean):	- (medium and solvent control), 0.72, 0.13, 0.26, 0.54, 1.2, 2.3 and 4.4 mg/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2), 0.1 mL/L
Test conditions:	
Test medium:	Algal Assay Procedure (AAP) medium (according to guideline), initial pH adjusted to 7.5 ± 0.1
Temperature:	23 - 24 °C
pH:	7.2 – 7.3 (0 h), 7.2 – 8.2 (72 h)
Conductivity:	84 - 120 µmhos/cm (0 h), 83 – 120 µmhos/cm (72 h)
Incubation:	Continuous illumination at 4500 to 5900 lux
Test parameters:	Cell counts were estimated using a haemocytometer and microscope. Observations of the health and morphology of the algal cells were made under the microscope on each study day. For chemical analysis (LC/MS/MS method) of test the substance, samples of test solution were taken at test initiation, after 72 h and at test termination. Measurements of pH and conductivity were made at initiation and at termination, light intensity was measured at daily intervals and temperature was monitored continuously.
Statistics:	Normal distribution and homogeneity of variance: Shapiro Wilks' Test and Bartlett's Test Significance of effects compared to the control: Bonferroni's adjusted t-Test (biomass), Dunnett's Multiple Comparison Test (growth rate)

Findings:

Analytical data:	Mean measured concentrations were in the range of 79 to 92% of nominal concentrations over the whole test duration.
Morphological effects:	No effects on the morphology and appearance of the cells were observed during the study period.
Biomass, growth rate	See Table 130 and 131

and cell density:

Table 150: Cell density of *P. subcapitata* after 24, 48 and 72 h of exposure to S-2167

S-2167 [mg/L] (mean measured)	**Cell density (x 10^4 cells/mL), (± SD)**		
	24 h	**48 h**	**72 h**
Control	5.42 (1.61)	20.50 (6.71)	82.75 (19.52)
Solvent control	5.38 (1.92)	23.88 (5.22)	94.38 (30.17)
0.072	5.42 (1.04)	25.50 (6.06)	77.25 (14.32)
0.13	5.75 (1.09)	20.67 (10.02)	67.58 (12.50)
0.26	5.33 (1.81)	13.58 (4.06)	58.25 (17.35)
0.54	4.25 (0.90)	9.00 (1.75)	32.42 (5.48)
1.2	2.33 (0.88)	9.08 (1.70)	18.00 (3.28)
2.3	2.67 (1.59)	4.92 (1.18)	9.33 (2.04)
4.4	1.58 (0.58)	3.67 (1.01)	7.42 (2.50)

Table 151: Effects of technical S-2167 on the green alga *P. subcapitata*

S-2167 [mg/L] (mean measured)	**Percent inhibition relative to the solvent control [%]**	
	Biomass (0 – 72 h)	**Growth rate (0 – 72 h)**
Control	-	-
Solvent control	-	-
0.072	9	3
0.13	22	7
0.26	38 **	11
0.54	64 **	23 *
1.2	76 **	36 *
2.3	87 **	51 *
4.4	91 **	56 *
NOEC	0.13 mg/L	0.26 mg/L
EC_{10}	0.074 mg/L (n.a. – 0.25 mg/L)	0.24 mg/L (n.a. – 0.48 mg/L)
EC_{20}	0.12 mg/L (n.a. – 0.41 mg/L)	0.47 mg/L (0.17 – 0.78 mg/L)
EC_{50} (95 % C.l.)	0.38 mg/L (0.06 – 0.60 mg/L)	2.2 mg/L (1.5 – n.a. mg/L)

* Significantly different compared to the solvent control, based on Dunnett's Multiple Comparison Test
** Significantly different compared to the solvent control, based on Bonferroni's adjusted t-Test
n.a… not applicable, corresponding 95% confidence limit could not be calculated.

Conclusion: 72 h E_bC_{50} = 0.38 mg/L
72 h E_rC_{50} = 2.2 mg/L
72 h NOEC = 0.13 mg/L (biomass)
72 h NOEC = 0.26 mg/L (growth rate)
based on mean measured concentrations.

Reference:	**S-2200: A 7-day static-renewal toxicity test with duckweed (*Lemna gibba* G3)**
Author(s), year:	Jacobs, Allison M., Porch, John R., Kendall, Timothy Z. and Krueger, Henry O., 2012a
Report/Doc. number:	Report No. ROW-0065, Project No. VP-38202
Guideline(s):	US EPA OPPTS 850.4400 (1996), OECD Guideline 221 (2006)
GLP:	Yes
Deviations:	None
Validity:	Acceptable

Material and methods:

Test substance:	S-2200 technical grade, purity: 93.4%, batch: ST-0811G
Reference substance:	S-2354 (S-2200 *S*-isomer), purity: 99.4%, batch: AS 2263a S-2167 (S-2200 *R*-isomer), purity: 99.8%, batch: AS 2262a
Test species:	Duckweed (*Lemna gibba* G3)
Number of organisms:	4 plants with a total of 12 fronds, 3 replicates per treatment and controls
Type of test, duration:	Static-renewal test, 7 days
Applied concentrations:	
Nominal:	0 (control and solvent control), 0.16, 0.31, 0.63, 1.3 and 2.5 mg ai/L
Measured (mean):	- (control and solvent control), 0.15, 0.32, 0.63, 1.2 and 2.3 mg ai/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Test conditions:	
Culture medium:	20X AAP medium, pH = 7.6
Temperature:	25 ± 2°C
pH:	8.1 – 8.3 (new solution), 8.7 – 9.0 (aged solution)
Light regime:	Continuous warm-white fluorescent lighting (~ 6500 lux)
Test parameters:	Growth, defined as an increase in the total number of fronds in each replicate test chamber, was determined through direct counts on Days 3 and 5 during the test and at the end of the test for all treatment and control replicates. In addition, the total number of duckweed plants in each replicate test chamber was determined at test termination. Observations of effects such as chlorosis, necrosis, dead fronds, root destruction and break-up of duckweed colonies were performed on Days 0, 3 and 5 during the test and at the end of the test. Any other abnormalities in frond or plant appearance were also documented. Biomass (dry weight in milligrams) was determined at the beginning of the test from three samples (12 fronds/4 plants) of the culture, and at the end of the exposure period with the plant material from each treatment and control replicate after completion of the final-day frond count. Measurements of temperature, pH and light: temperatures remained within the 25 ± 2°C range established for the test, with the exception of one measurement that was slightly out of range at 22.8°C; the pH in the new test solutions ranged from 8.1 to 8.3 and ranged from 8.7 to 9.0 in the old test solutions; the light intensity ranged from 5,750 to 6,060 lux, which was within the desired range of 6,500 lux ± 15%. Test solution samples were collected from each treatment and control group at test initiation, on Day 3 and 5 and at test termination. Samples of new solutions were collected on Days 0, 3, and 5 from the individual batches of test solution prepared for each treatment and control group

prior to distribution to the test chambers. Samples of the old solutions were collected prior to renewal on Day 3, 5 and at test termination on Day 7 from the pooled replicates of each treatment and control group. All samples were analyzed by high performance liquid chromatography with ultraviolet detection (HPLC/UV).

Statistics: Day 7 EC_{50} values were estimated, when possible, using non-linear regression with treatment response (frond number, final biomass, and growth rates based on frond number and biomass) and exposure concentration data. The percentages of dead, chlorotic and necrotic fronds were calculated as the number of dead, chlorotic and necrotic fronds relative to the total number of fronds present in each replicate test chamber. The Day 7 frond number, final biomass and growth rate data were evaluated for normality and homogeneity of variances (α = 0.01) using the Shapiro-Wilk's and Levene's tests, respectively. All data met the assumptions for normal distribution and homogeneity of variance. Negative and solvent control group data for frond number, final biomass and growth rate were compared with a t-test (α = 0.05) in order to determine if significant differences were observed. There were no significant differences therefore, treatment group means were compared to the negative control mean (α = 0.05) using analysis of variance (ANOVA) and Dunnett's t-test. Results of the statistical analyses, as well as an evaluation of the concentration-response pattern and other observations of effects, were used in the determination of the no observed adverse effect concentration (NOAEC) and lowest observed effect concentration (LOEC).

Findings:

Analytical data: Measured concentrations of S-2200 ranged from 94 to 101% of nominal in samples collected on Day 0, from 84-107% of nominal on Day 3, from 82-104% of nominal on Day 5 and from 94 to 103% of nominal at test termination on Day 7. Overall, the mean measured concentrations compared to the nominal concentrations were between 92 and 103%.

Table: 152: Concentrations measured during the 7 d static-renwal exposure of duckweed to S-2200 technical grade

S-2200 [mg ai/L] (nominal)	**Measured concentration [mg ai/L]**							**Percent of nominal [%]**
	0-hour			**7 days**			**Mean**	
	S-2167	**S-2354**	**S-2200**	**S-2167**	**S-2354**	**S-2200**		
Control	-	-	< LOQ	-	-	< LOQ	n.a.	n.a.
Solvent control	-	-	< LOQ	-	-	< LOQ	n.a.	n.a.
0.16	0.0730	0.0769	0.150	0.0764	0.0783	0.155	0.15	94
0.31	0.155	0.157	0.312	0.156	0.161	0.317	0.32	103
0.63	0.314	0.318	0.632	0.320	0.327	0.647	0.63	100
1.3	0.615	0.621	1.24	0.640	0.649	1.29	1.2	92
2.5	1.19	1.21	2.40	1.17	1.18	2.35	2.3	92

n.a…not applicable, LOQ…Limit of quantification (0.08 mg ai/L)
Concentrations expressed as less than values were below the minimum detectable limit (MDL).

Biological effects: At the end of the study (day 7) chlorotic and necrotic effects were observed in the negative control and at a test concentration of 0.63 and 2.5 mg ai/L.

However, the observed effects were only slight, below 1% of the average percentage for the replicates per treatment.

Table 153: Mean frond number, final biomass and percent inhibition

S-2200 [mg ai/L] (mean measured)	Mean frond number [a] (percent inhibition relative to the negative control)			
	Day 3	Day 5	Day 7	Mean final biomass[b]
Control	26 (-)	57 (-)	135 (-)	19.9 (-)
Solvent control	24 (9)	56 (1)	124 (8)	18.9 (5)
0.15	26 (1)	56 (2)	125 (8)	18.8 (6)
0.32	26 (1)	56 (2)	130 (4)	19.5 (2)
0.63	24 (6)	51 (9)	115 (15) *	18.0 (10)
1.2	24 (8)	48 (15)	96 (29) *	17.2 (14)
2.3	23 (12)	38 (33)	68 (50) *	13.8 (31) *

[a] Mean number of fronds per treatment and control group on day 0 = 12 (per replicate)
[b] Final biomass was calculated based on Day 7 biomass in each replicate minus Day 0 biomass of a representative sample from the culture.
* Statistically significant reductions from the negative control, Dunnett's t-test ($p \leq 0.05$)

Table 134: Mean frond number, biomass growth rate and percent inhibition

S-2200 [mg ai/L] (mean measured)	Mean frond number growth rate (percent inihibtion relative to the negative control)			Mean biomass growth rate (percent inihibtion relative to the negative control)
	Day 0-3	Day 0-5	Day 0-7	Day 0-7
Control	0.2566 (-)	0.3099 (-)	0.3456 (-)	0.4255 (-)
Solvent control	0.2263 (12)	0.3088 (0)	0.3335 (3)	0.4186 (2)
0.15	0.2532 (1)	0.3067 (1)	0.3339 (3)	9,4176 (2)
0.32	0.2527 (2)	0.3065 (1)	0.3399 (2)	0.4225 (1)
0.63	0.2345 (9)	0.2905 (6)	0.3224 (7) *	0.4119 (3)
1.2	0.2310 (10)	0.2786 (10)	0.2965 (14) *	0.4057 (5)
2.3	0.2167 (16)	0.2299 (26)	0.2473 (28) *	0.3762 (12) *

[a] Mean number of fronds per treatment and control group on day 0 = 12 (per replicate)
* Statistically significant reductions from the negative control, Dunnett's t-test ($p \leq 0.05$)

Conclusion: 7 d EC_{50} > 2.3 mg ai/L (frond number, frond number growth rate, final biomass and biomass growth rate) based on mean measured concentrations. The NOEC and LOEC based on frond number and frond number growth rate were 0.32 and 0.63 mg a.i./L, respectively. The NOEC and LOEC based on final biomass and biomass growth rate were 1.2 and 2.3 mg a.i./L, respectively.

5.4.4 Other aquatic organisms (including sediment)

Not relevant

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

Endpoint	Classification Criteria - CLP (2nd ATP) (criteria in bold)	Evidence for Mandestrobin
Degradation Mandestrobin	Hydrolotic degradataion of Mandestrobin pH 4: stable at 50°C pH 7: stable at 50 °C pH 9: stable at 50 °C Photodegradation of Mandestrobin was fast with an experimental half-life of 4.4 – 4.6 days under the test conditions. Mandestrobin is not readily biodegradable, and it does not meet the criterion for rapid degradation in a water/sediment study with a DT50 whole system of 332 days. Based on available data a non-rapid degradation is proposed for Mandestrobin.	The active substance is **not considered as ready biodegradable/rapid degradable.**
Bioaccumulation Mandestrobin	**Log K_{ow} is < 4** Mandestrobin Log K_{ow} =3.51 at 25 °C	The measured log P_{OW} is 3.51 (at 25 °C, mixture of both isomers) and is below the classification criteria of 4, therefore Mandestrobin is considered to have **a moderate bioaccumulation potential.**
Acute aquatic toxicity Mandestrobin	**E_rC_{50} > 1 mg/L (algae)** **LC_{50} < 1 mg/L (fish, marine and freshwater invertebrates)**	The classification is based on data with Mandestrobin (S-2200, 50:50 mixture of *S*- and *R*-isomer) and the biological active *R*-isomer (S-2167). Mandestrobin is of moderate toxicity to green algae (E_rC_{50} = 2.2 mg/L) and of high toxicity to fish and aquatic invertebrates (LC_{50} < 1 mg/L) and fulfills the criteria for the proposed classification as **H400** according to Regulation EC 1272/2008 are also met.
Chronic aquatic toxicity Mandestrobin	**For not rapidly degradable substances: NOEC ≤ 0.1 mg/L**	Mandestrobin is of high chronic toxicity to aquatic invertebrates (marine species) with a NOEC = 0.0056 mg/L. Therefore Mandestrobin fulfills the criteria for the proposed classification as **H410** according to Regulation EC 1272/2008.
	NOEC = 0.0056 mg/L (*Americamysis bahia)*	
SUMMARY	**H400 / H410**	**PROPOSED CLASSIFICATION**

Conclusion of environmental classification according to Regulation EC 1272/2008

Pictogram: GHS 09

Signal word: Warning!

Aquatic Acute 1 – H400 'Very toxic to aquatic life'

Aquatic Chronic 1 - H410 'Very toxic to aquatic life with long lasting effects'

M factor = 1 (acute) and 10 (chronic)

Justification for the proposal

H400 follows from the toxicity of the active substance S-2200 to fish (*Oncorhynchus mykiss*, LC_{50} = 0.94 mg/L, Fournier, A.E., 2009a) and aquatic invertebrates (*Americamysis bahia*, LC_{50} = 0.43 mg/L, Thomas et al., 2012).

H410 follows from the chronic toxicity of the active substance Mandestrobin to aquatic invertebrates (*Americamysis bahia*, NOEC = 0.0056 mg/L, Claude et al., 2012) and the fact that the active substance is not readily biodegradable (Graham, R., 2009) and not rapidly degradable (Graham, R., 2011ab). In the water-sediment study a DT_{50} of 212 - 519 days (range) was determined for the whole system.
Based on the fish bioaccumulation study (Lentz, N.R., 2010) with *L. macrochirus* a steady-state BCF (whole fish) of 25-26 was determined, which indicate a low potential to bioaccumulate in the aquatic food chain. The substance Mandestrobin does not meet the CLP criteria (BCF ≥ 500) based on the measured fish BCF.

Mandestrobin fulfils the criteria for classification as aquatic environmental hazard based on the CLP Regulation and should be classified.

The statements **P273**, **P391** and **P501** follow a general precautionary approach for dangerous substances.

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

Mandestrobin was hydrolytically stable at pH 4, 7 and 9 at 50°C. According to OECD 111 the expected DT_{50} at 25°C would be >1 year for each isomer and hence for the racemate, S-2200. No hydrolysis of S-2167 and of S-2354 would be expected under environmental conditions.

Mandestrobin is not readily biodegradable and it cannot be classified as rapidly degraded in water sediment systems since less than 70 % is degraded within 28 days ($DT_{50whole\ system}$ > 100 days). Furthermore, mineralisation of the active substance is below 10 % of AR after 100 days after application.

Mandestrobin has a low potential of bioaccumulation in aquatic system because of a measured fish BCF of 25-26 (Lentz, N.R., 2010).

Mandestrobin is acute toxic to fish (*Oncorhynchus mykiss*, LC_{50} = 0.94 mg/L, Fournier, A.E., 2009a) and aquatic invertebrates (*Americamysis bahia*, LC_{50} = 0.43 mg/L, Thomas et al., 2012). Mandestrobin is chronic toxic to aquatic invertebrates (*Americamysis bahia*, NOEC = 0.0056 mg/L, Claude et al., 2012).

Hazard pictogram		Environment
Hazard class and category:	Hazardous to the aquatic environment, Acute Hazard Category 1 Hazardous to the aquatic environment, Chronic Hazard Category 1	
Signal word	Warning!	
Hazard statement:	H400 H410	Very toxic to aquatic life Very toxic to aquatic life with long lasting effects
Precautionary statements - Prevention	P273	Avoid release to the environment
Precautionary statements - Response	P391	Collect spillage
Precautionary Statement Disposal	P501	Proper disposal of contents/container

6 OTHER INFORMATION

7 REFERENCES

7.1 Physico-chemical properties

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Foster, B.	2011	S-2200TG: Determination of Oxidising Properties Covance Laboratories Ltd, Study No: 8245520 Sumitomo Chemical Co., Ltd. Report ROP-0023 GLP, Unpublished	Y	SUM
Foster, B.	2012a	S-2200TG: Determination of Physical State, Colour and Odour and Relative Density Smithers Viscient Study no. 8262798 Sumitomo Chemical Co., Ltd. ROP-0039 GLP, Unpublished	Y	SUM
Foster, B.	2012b	S-2200TG: Determination of Surface Tension Covance Laboratories Ltd, Study No: 8245521 Sumitomo Chemical Co., Ltd. Report ROP-0026 GLP, Unpublished	Y	SUM
Foster, B. & Heslop, D.	2012	S-2200TG: Determination of Flammability Covance Laboratories Ltd, Study No: 8245519 Sumitomo Chemical Co., Ltd. Report ROP-0025 GLP, Unpublished	Y	SUM
Lentz, N.R.	2010	S-2200 TGAI – Determining the Auto-Flammability (Temperature of Self Ignition of Solids) Following the Official Journal of the European Communities L383A, Method A.16 Springborn Smithers Laboratories Report no. 13048.6615 Sumitomo Chemical Co., Ltd. ROP-0008 GLP, Unpublished	Y	SUM
Lentz, N.R. & Van Meter, D.S	2010a	S-2200 TGAI – Determination of the Solvent Solubility Smithers Viscient Report no. 13048.6609 Sumitomo Chemical Co., Ltd. ROP-0012 GLP, Unpublished	Y	SUM
Lentz, N.R. & Van Meter, D.S	2010b	Determination of the Impact Explodability of S-2200 TGAI following OPPTS Guideline 830.6316 Springborn Smithers Laboratories Report no. 13048.6646 Sumitomo Chemical Co., Ltd. ROP-0007 GLP, Unpublished	Y	SUM
Lentz, N.R. & Van Meter, D.S	2011a	S-2200 PAI – Determination of the Solvent Solubility Smithers Viscient Report no. 13048.6610 Sumitomo Chemical Co., Ltd. ROP-0014 GLP, Unpublished	Y	SUM

Lentz, N.R. & Van Meter, D.S	2011b	S-2167 PAI – Determination of the Solvent Solubility Smithers Viscient Report no. 13048.6611 Sumitomo Chemical Co., Ltd. ROP-0015 GLP, Unpublished	Y	SUM
Lentz, N.R. & Van Meter, D.S	2011c	S-2354 PAI – Determination of the Solvent Solubility Smithers Viscient Report no. 13048.6612 Sumitomo Chemical Co., Ltd. ROP-0016 GLP, Unpublished	Y	SUM
Lentz, N.R. & Van Meter, D.S.	2009a	Determination of the Water Solubility of S-2200 PAI Springborn Smithers Laboratories Report no. 13048.6606 Sumitomo Chemical Co., Ltd. ROP-0001 GLP, Unpublished	Y	SUM
Lentz, N.R. & Van Meter, D.S.	2009b	Determination of the Water Solubility of S-2167 PAI Springborn Smithers Laboratories Report no. 13048.6607 Sumitomo Chemical Co., Ltd. ROP-0002 GLP, Unpublished	Y	SUM
Lentz, N.R. & Van Meter, D.S.	2009c	Determination of the Water Solubility of S-2354 PAI Springborn Smithers Laboratories Report no. 13048.6608 Sumitomo Chemical Co., Ltd. ROP-0003 GLP, Unpublished	Y	SUM
Lewis, C. J. & Alderman, D.	2010b	[^{14}C]S-2354 (S-2200 *S*-isomer): Hydrolytic Stability Covance Laboratories Ltd, Study No: 8200194 Sumitomo Chemical Co., Ltd. Report ROM-0006 GLP, Unpublished.	Y	SUM
Lewis, C. J. & Alderman, D.	2010c	[^{14}C]S-2167 (S-2200 *R*-isomer): Photodegradation and Quantum Yield in Sterile, Aqueous Solution Covance Laboratories Ltd, Study No: 8200199 Sumitomo Chemical Co., Ltd. Report ROM-0013 GLP, Unpublished.	Y	SUM
Lewis, C. J. & Alderman, D.	2010d	[^{14}C]S-2354 (S-2200 *S*-isomer): Photodegradation and Quantum Yield in Sterile, Aqueous Solution. Amended Final Report 1 Covance Laboratories Ltd, Study No: 8200195 Sumitomo Chemical Co., Ltd. Report ROM-0011 GLP, Unpublished.	Y	SUM
Lewis, C.J. & Alderman, D.	2010a	[^{14}C]S-2167 (S-2200 *R*-isomer): Hydrolytic Stability Covance Laboratories Ltd, Study No: 8200206 Sumitomo Chemical Co., Ltd. Report ROM-0005 GLP, Unpublished.	Y	SUM
Liney, P. & Jarvis, T.	2012a	S-2200 – Henry's Law Constant Exponent International Ltd Sumitomo Chemical Co., Ltd. ROP-0034 Non-GLP, Unpublished	N	SUM

Liney, P. & Jarvis, T.	2012b	S-2200 – Stability in Air Exponent International Ltd Sumitomo Chemical Co., Ltd. ROP-0035 Non-GLP, Unpublished	Y	SUM
Nishimura, H., Fujisawa, T., Katagi, T.	2012	S-2200: Calculation of Aqueous Photolysis Rates in Near Surface Water at North Latitudes 10° to 80° using GCSOLAR Programme Sumitomo Chemical Co., Ltd. ROP-0032 Non-GLP, Unpublished.	Y	SUM
Proctor, K.L. & Lentz, N.R.	2011a	S-2200 PAI – Determination of Vapour Pressure Smithers Viscient Report no. 13048.6604 Sumitomo Chemical Co., Ltd. ROP-0021 GLP, Unpublished	Y	SUM
Proctor, K.L. & Lentz, N.R.	2011b	S-2167 PAI – Determination of Vapour Pressure Smithers Viscient Report no. 13048.6605 Sumitomo Chemical Co., Ltd. ROP-0022 GLP, Unpublished	Y	SUM
Van Meter, D.S. & Lentz, N.R.	2010a	Product Chemistry Testing for S-2200 PAI Springborn Smithers Laboratories Report no. 13048.6601 Sumitomo Chemical Co., Ltd. ROP-0009 GLP, Unpublished	Y	SUM
Van Meter, D.S. & Lentz, N.R.	2010b	Product Chemistry Testing for S-2167 PAI Springborn Smithers Laboratories Report no. 13048.6602 Sumitomo Chemical Co., Ltd. ROP-0010 GLP, Unpublished	Y	SUM
Van Meter, D.S. & Lentz, N.R.	2010c	Product Chemistry Testing for S-2354 PAI Springborn Smithers Laboratories Report no. 13048.6603 Sumitomo Chemical Co., Ltd. ROP-0011 GLP, Unpublished	Y	SUM
Van Meter, D.S. & Lentz, N.R.	2010d	Determination of the Partition Coefficient (*n*-Octanol/Water) – S-2200 PAI Springborn Smithers Laboratories Report no. 13048.6613 Sumitomo Chemical Co., Ltd. ROP-0005 GLP, Unpublished	Y	SUM
Van Meter, D.S. & Lentz, N.R.	2010e	Determination of the Partition Coefficient (*n*-Octanol/Water) – S-2167 PAI Springborn Smithers Laboratories Report no. 13048.6614 Sumitomo Chemical Co., Ltd. ROP-0004 GLP, Unpublished	Y	SUM
Van Meter, D.S. & Lentz, N.R.	2011	Product Chemistry Testing for S-2200 TGAI Smithers Viscient Report no. 13048.6636 Sumitomo Chemical Co., Ltd. ROP-0013 GLP, Unpublished	Y	SUM

7.2 Human health hazard assessment

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Kendrick, J., Farrell, C. & Murphy, B.	2012	Amended Final Report 1. [^{14}C]S-2200: Absorption, Distribution, Metabolism and Excretion following Single Oral Administration to the Rat. Covance Laboratories Ltd., Study No. 0333/297. (Sumitomo ROM-0033) GLP, Unpublished.	Y	SUM
Mikata, K.	2011	Metabolism of S-2200 R-isomer (S-2167) and S-2200 S-isomer (S-2354) in Rats. Sumitomo, Study No. 4136. (Sumitomo ROM-0021) GLP, Unpublished.	Y	SUM
Kendrick, J. & Farrell, C.	2012	Amended Final Report 1. [^{14}C]S-2200: Absorption, Distribution, Metabolism and Excretion following Repeat Oral Administration to the Rat Covance Laboratories Ltd., Study No. 0333/298. (Sumitomo ROM-0036) GLP, Unpublished.	Y	SUM
Asano, H.	2010a	Acute Oral Toxicity Study of S-2200TG in Rats Sumitomo, Study No. 4161 (Sumitomo ROT-0010) GLP, Unpublished.	Y	SUM
Asano, H.	2010b	Acute Dermal Toxicity Study of S-2200TG in Rats Sumitomo, Study No. 4162 (Sumitomo ROT-0011) GLP, Unpublished.	Y	SUM
Deguchi, Y.	2010	Acute Inhalation Toxicity Study of S-2200 TG in Rats Sumitomo, Study No. 4160 (Sumitomo ROT-0020) GLP, Unpublished.	Y	SUM
Ota, M.	2010a	Primary Skin Irritation test of S-2200TG in Rabbits Sumitomo, Study No. 4152 (Sumitomo ROT-0015) GLP, Unpublished.	Y	SUM
Ota, M.	2010b	Amended Final Report: Primary Eye Irritation Test of S-2200TG in Rabbits Sumitomo, Study No. 4153 (Sumitomo ROT-0016) GLP, Unpublished.	Y	SUM
Ota, M.	2010c	Skin Sensitization Test of S-2200TG in Guinea Pigs (Maximization Test) Sumitomo, Study No. 4165 (Sumitomo ROT-0019) GLP, Unpublished.	Y	SUM

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Beck, W.	2011a	S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Rat Covance Laboratories Ltd., Study No. 0333/290 (Sumitomo ROT-0025) GLP, Unpublished.	Y	SUM
Beck, W.	2011b	Amended Final Report 1: S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Mouse Covance Laboratories Ltd., Study No. 0333/291 (Sumitomo ROT-0023) GLP, Unpublished.	Y	SUM
Beck, W.	2012d	Amended Final Report 2: S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Dog Covance Laboratories Ltd., Study No. 8211560 (Sumitomo ROT-0024) GLP, Unpublished.	Y	SUM
Beck W	2012a	S-2200 Technical Grade: 52 Week Oral (Dietary) Administration Toxicity Study in the Dog Covance Laboratories Ltd., Study No. 8211561 (Sumitomo ROT-0071) GLP, Unpublished.	Y	SUM
Ogata, H.	2011	A 28-Day Repeated Dose Dermal Toxicity Study of S-2200 Technical Grade in Rats Mitsubishi Chemical Medience Corporation, Study No. P100595 (Sumitomo ROT-0022) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2010a	Reverse Mutation Test of S-2200TG in Bacterial Systems Sumitomo, Study No. 4163. (Sumitomo ROT-0012) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2010b	*In vitro* Chromosomal Aberration Test on S-2200TG in Chinese Hamster Lung Cells (CHL/IU) Sumitomo, Study No. 4164 (Sumitomo ROT-0013) GLP, Unpublished.	Y	SUM
Wollny, H-E.	2010	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with S-2200TG Harlan Cytotest Cell Research GmbH, Study No. 1289600 (Sumitomo ROT-0021) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2010c	Micronucleus Test on S-2200TG in CD-1 Mice. Sumitomo, Study No. 4154 (Sumitomo ROT-0014) GLP, Unpublished.	Y	SUM

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Beck, W.	2012b	S-2200 Technical Grade: 104 Week Oral (Dietary) Administration Combined Toxicity/Carcinogenicity Study in the Rat. Covance Laboratories Ltd., Study No. 0333/292 (Sumitomo ROT-0072) GLP, Unpublished.	Y	SUM
Beck, W	2012c	S-2200 Technical Grade: 78 Week Oral (Dietary) Administration Carcinogenicity Study in the Mouse. Covance Laboratories Ltd., Study No. 0333/293 (Sumitomo ROT-0073) GLP, Unpublished.	Y	SUM
Yamada, T.	2012a	The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action Sumitomo, Study No. none (Sumitomo ROT-0070) Non-GLP, Unpublished.	Y	SUM
Asano, H.	2012e	Short-Term Study for Mode of Action Analysis for Rat Liver and Thyroid Findings by S-2200TG –Dose Response, Time-Course and Reversibility. Sumitomo, Study No. S1560 (Sumitomo ROT-0067) Non-GLP, Unpublished.	Y	SUM
Yamada, T.	2012b	Short-Term Study for Mode of Action Analysis for Mouse Liver Findings by S-2200TG. Sumitomo, Study No. S1618 (Sumitomo ROT-0068) Non-GLP, Unpublished.	Y	SUM
Yamada, T. & Miyata, K.	2012	Interpretation of Higher Incidence of Ovarian Sex-cord Stromal Tumour in Female Rats treated with S-2200TG in a 2-year carcinogenicity study. Sumitomo, Study No. none (Sumitomo ROT-0069) Non-GLP, Unpublished.	Y	SUM
Kubo, H.	2012	*In vitro* Steroidogenesis Assay of S-2200TG in H295R Cells Sumitomo, Study No. HK001 (Sumitomo ROT-0065) Non-GLP, Unpublished.	Y	SUM
Suzuki, N.	2012	Evaluation of Effects of S-2200TG and its Metabolites on Human Estrogen Receptor alpha and Human Androgen Receptor using *in vitro* Reporter Gene Assays Sumitomo, Study No. RGA-130 (Sumitomo ROT-0066) Non-GLP, Unpublished.	Y	SUM

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Yamada., T.	2013	Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study Sumitomo, Study No. none (Sumitomo ROT-0075) Non-GLP, Unpublished.	Y	SUM
Hoshino, N.	2010	Dose Range-finding Study for Two Generation Reproduction Study of S-2200 TG in Rats. Mitsubishi Chemical Medience Corporation, Study No. B091037 (Sumitomo ROT-0018) Non-GLP, Unpublished.	Y	SUM
Matsuura, I.	2012	Two-Generation Reproduction Toxicity Study of S-2200TG in Rats Mitsubishi Chemical Medience Corporation, Study No. B100842 (Sumitomo ROT-0064) GLP, Unpublished.	Y	SUM
Rhodes, J.	2009a	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rat Covance Laboratories Ltd., Study No. 8202048 (Sumitomo ROT-0009) Non- GLP, Unpublished.	Y	SUM
Rhodes, J.	2012a	S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rat Covance Laboratories Ltd., Study No. 8202037 (Sumitomo ROT-0051) GLP, Unpublished.	Y	SUM
Rhodes, J.	2009b	S-2200 TG: Oral (Gavage) Range-Finding Study of Prenatal Development in the Rabbit Covance Laboratories Ltd., Study No. 8202040 (Sumitomo ROT-0008) Non-GLP, Unpublished.	Y	SUM
Rhodes, J.	2012b	S-2200 TG: Oral (Gavage) Prenatal Development Toxicity Study in the Rabbit Covance Laboratories Ltd., Study No. 8202046 (Sumitomo ROT-0052) GLP, Unpublished.	Y	SUM
Herberth, M.T.	2011a	An Oral (Gavage) Dose Range-Finding Acute Neurotoxicity Study of S-2200TG in Wistar Rats WIL Research Laboratories, LLC, Study No. WIL-118051 (Sumitomo ROT-0036) GLP, Unpublished.	Y	SUM
Herberth, M.T.	2011b	An Oral (Gavage) Acute Neurotoxicity Study of S-2200TG in Wistar Rats. WIL Research Laboratories, LLC, Study No. WIL-118052 (Sumitomo ROT-0037) GLP, Unpublished.	Y	SUM

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Herberth, M.T.	2012	A 90-Day Oral Dietary Neurotoxicity Study of S-2200TG in Wistar Rats WIL Research Laboratories, LLC, Study No. WIL-118053 (Sumitomo ROT-0050) GLP, Unpublished.	Y	SUM
Asano, H.	2012a	Acute Oral Toxicity Study of 2-COOH-S-2200 in Rats Sumitomo, Study No. 4234 (Sumitomo ROT-0043) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2012a	Reverse Mutation Test of 2-COOH-S-2200 in Bacterial Systems Sumitomo, Study No. 4221. (Sumitomo ROT-0041) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2012b	*In vitro* Chromosomal Aberration Test on 2-COOH-S-2200 in Chinese Hamster Lung Cells (CHL/IU) Sumitomo, Study No. 4219. (Sumitomo ROT-0046) GLP, Unpublished.	Y	SUM
Wollny, H-E.	2011a	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with 2-COOH-S-2200 Harlan Cytotest Cell Research GmbH, Study No. 1394801. (Sumitomo ROT-0033) GLP, Unpublished.	Y	SUM
Tanaka, J.	2012	Micronucleus Assay of 2-COOH-S-2200 in Mice BSRC, Study No. D624 (028-071). (Sumitomo ROT-0040) GLP, Unpublished.	Y	SUM
Asano, H.	2012b	Acute Oral Toxicity Study of 5-COOH-S-2200 in Rats Sumitomo, Study No. 4233 (Sumitomo ROT-0044) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2012c	Reverse Mutation Test of 5-COOH-S-2200 in Bacterial Systems Sumitomo, Study No. 4222. (Sumitomo ROT-0042) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2012d	*In vitro* Chromosomal Aberration Test on 5-COOH-S-2200 in Chinese Hamster Lung Cells (CHL/IU) Sumitomo, Study No. 4220. (Sumitomo ROT-0047) GLP, Unpublished.	Y	SUM
Wollny, H-E.	2011b	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with 5-COOH-S-2200 Harlan Cytotest Cell Research GmbH, Study No. 1394802. (Sumitomo ROT-0034) GLP, Unpublished.	Y	SUM

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Asano, H.	2012c	Acute Oral Toxicity Study of 2-CH_2OH-S-2200 in Rats Sumitomo, Study No. 4242 (Sumitomo ROT-0053) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2012e	Reverse Mutation Test of 2-CH_2OH-S-2200 in Bacterial Systems Sumitomo, Study No. 4226. (Sumitomo ROT-0048) GLP, Unpublished.	Y	SUM
Asano, H.	2012d	Acute Oral Toxicity Study of 4-OH-S-2200 in Rats Sumitomo, Study No. 4241 (Sumitomo ROT-0054) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2012f	Reverse Mutation Test of 4-OH-S-2200 in Bacterial Systems Sumitomo, Study No. 4227. (Sumitomo ROT-0049) GLP, Unpublished.	Y	SUM
Asano, H.	2011	Acute Oral Toxicity Study of De-Xy-S-2200 in Rats Sumitomo, Study No. 4225 (Sumitomo ROT-0031) GLP, Unpublished.	Y	SUM
Kitamoto, S.	2011	Reverse Mutation Test of De-Xy-S-2200 in Bacterial Systems Sumitomo, Study No. 4223. (Sumitomo ROT-0038) GLP, Unpublished.	Y	SUM
Nishioka, K.	2012	Statement from S-2200 Manufacturer Sumitomo, Study No. none (Sumitomo ROT-0074) Non-GLP, Unpublished	Y	SUM
Hosako, H.	2011a	S-2200TG – A 28-Day Dietary Dose Range-Finding Study in Wistar Han Rats WIL Research Laboratories, LLC, Study No. WIL-118055 (Sumitomo ROT-0035) Non-GLP, Unpublished.	Y	SUM
Hosako, H.	2011b	S-2200TG – A 28-Day Oral (Dietary) Immunotoxicity Study in Female Wistar Han Rats WIL Research Laboratories, LLC, Study No. WIL-118056 (Sumitomo ROT-0039) GLP, Unpublished.	Y	SUM

7.3 Environmental hazard assessment

7.3.1 Fate and Behaviour in the environment

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Graham, R.	2009	S-2200 (Racemic mixture): Assessment of Ready Biodegradability by Measurement of CO_2 Evolution Covance Laboratories Ltd, Sumitomo Chemical Co., Ltd. Report ROM-0003 GLP, Unpublished.	Y	SUM
Graham, R.	2011a	[^{14}C]S-2167 (S-2200 *R*-isomer): Degradation in Water-Sediment Systems under Aerobic Conditions Covance Laboratories Ltd, Sumitomo Chemical Co., Ltd. Report ROM-0022 GLP, Unpublished.	Y	SUM
Graham, R.	2011b	[^{14}C]S-2354 (S-2200 *S*-isomer): Degradation in Water-Sediment Systems under Aerobic Conditions. Amended Final Report Covance Laboratories Ltd, Sumitomo Chemical Co., Ltd. Report ROM-0023 GLP, Unpublished.	Y	SUM
Jarvis, T. & Mamouni, A	2012	Calculation of S-2200 sediment water kinetics according to FOCUS (2006) Guidance Exponent International Ltd Sumitomo Chemical Co., Ltd. Report ROM-0034 Non-GLP, Unpublished.	Y	SUM
Lewis, C.J. & Alderman, D.	2010a	[^{14}C]S-2167 (S-2200 *R*-isomer): Hydrolytic Stability Covance Laboratories Ltd, Sumitomo Chemical Co., Ltd. Report ROM-0005 GLP, Unpublished.	Y	SUM
Lewis, C. J. & Alderman, D.	2010b	[^{14}C]S-2354 (S-2200 *S*-isomer): Hydrolytic Stability Covance Laboratories Ltd, Sumitomo Chemical Co., Ltd. Report ROM-0006 GLP, Unpublished.	Y	SUM
Lewis, C. J. & Alderman, D.	2010c	[^{14}C]S-2167 (S-2200 *R*-isomer): Photodegradation and Quantum Yield in Sterile, Aqueous Solution Covance Laboratories Ltd, Sumitomo Chemical Co., Ltd. Report ROM-0013 GLP, Unpublished.	Y	SUM
Lewis, C. J. & Alderman, D.	2010d	[^{14}C]S-2354 (S-2200 *S*-isomer): Photodegradation and Quantum Yield in Sterile, Aqueous Solution. Amended Final Report 1 Covance Laboratories Ltd, Sumitomo Chemical Co., Ltd. Report ROM-0011 GLP, Unpublished.	Y	SUM

7.3.2 Aquatic Toxicity

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
Claude, M.B., Kendall, T.Z., Gallagher, S.P. & Krueger, H.O.	2012	S-2200: A flow-through life-cycle toxicity test with the saltwater mysid (*Americamysis bahia*) Wildlife International, Ltd., Report No. 263A-140 (Sumitomo ROW-0063) GLP, Unpublished	Y	SUM
Fournier, A.E.	2009a	S-2200 Technical Grade - Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions, Following OECD Guideline #203, EC Guideline L383A, Method C.1 and OPPTS Draft Guideline 850.1075 Springborn Smithers Laboratories Study No. 13048.6622 (Sumitomo ROW-0007) GLP, Unpublished	Y	SUM
Fournier, A.E.	2009b	S-2354 (S-Isomer of S-2200) - Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions, Following OECD Guideline #203, EC Guideline L383A, Method C.1 and OPPTS Draft Guideline 850.1075 Springborn Smithers Laboratories Study No. 13048.6621 (Sumitomo ROW-0011) GLP, Unpublished	Y	SUM
Fournier, A.E.	2009c	S-2167 (R-Isomer of S-2200) - Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions, Following OECD Guideline #203, EC Guideline L383A, Method C.1 and OPPTS Draft Guideline 850.1075 Springborn Smithers Laboratories Study No. 13048.6623 (Sumitomo ROW-0010) GLP, Unpublished	Y	SUM
Fournier, A.E.	2009d	S-2200 Technical Grade - Acute Toxicity to Bluegill Sunfish (*Lepomis macrochirus*) Under Static Conditions, Following OECD Guideline #203, EC Guideline L383A, Method C.1 and OPPTS Draft Guideline 850.1075 Springborn Smithers Laboratories Study No. 13048.6624 (Sumitomo ROW-0008) GLP, Unpublished	Y	SUM
Fournier, A.E.	2009e	S-2200 Technical Grade - Acute Toxicity to Fathead Minnow (*Pimephales promelas*) Under Static Conditions, Following OECD Guideline #203, EC Guideline L383A, Method C.1 and OPPTS Draft Guideline 850.1075 Springborn Smithers Laboratories Study No. 13048.6625 (Sumitomo ROW-0009) GLP, Unpublished	Y	SUM
Fournier, A.E.	2012e	S-2167 (R-Isomer of S-2200) - Acute Toxicity to Water Fleas (*Daphnia magna*) Under Static Conditions, Following OECD Guideline #202 and The Official Journal of the European Communities L 142/456, Method C.2	Y	SUM

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
		Smithers Viscient Study No. 13048.6704 (Sumitomo ROW-0048) GLP, Unpublished		
Fournier, A.E.	2012f	S-2354 (S-Isomer of S-2200) - Acute Toxicity to Water Fleas (*Daphnia magna*) Under Static Conditions, Following OECD Guideline #202 and The Official Journal of the European Communities L 142/456, Method C.2 Smithers Viscient Study No. 13048.6706 (Sumitomo ROW-0049) GLP, Unpublished	Y	SUM
Jacobs, A.M., Porch, J.R., Kendall, T.Z. & Krueger, H.O.	2012a	S-2200: A 7-day static-renewal toxicity test with duckweed (*Lemna gibba* G3) Wildlife International, Ltd., Report No. 263A-144 (Sumitomo ROW-0065) GLP, Unpublished	Y	SUM
Lee, M.R.	2010	S-2200 Technical Grade – Early Life-Stage Toxicity Test with Fathead Minnow, *Pimephales promelas,* Following OECD Guideline #210 and OPPTS Draft Guideline 850.1400 Springborn Smithers Laboratories Study No. 13048.6626 (Sumitomo ROW-0019) GLP, Unpublished	Y	SUM
Lentz, N.R.	2010	Flow-Through Bioconcentration and Metabolism Study of [^{14}C]S-2200 with Bluegill Sunfish (*Lepomis macrochirus*) Smithers Viscient Study No. 13048.6627 (Sumitomo ROM-0016) GLP, Unpublished	Y	SUM
Minderhout, T., Kendall, T.Z. & Gallagher, S.P	2012	S-2200: An early life-stage toxicity test with the Sheepshead minnow (*Cyprinodon variegatus*) Wildlife International, Ltd., Report No. 263A-139 (Sumitomo ROW-0061) GLP, Unpublished	Y	SUM
Picard, C.R.	2012	S-2200 – Toxicity Test with Sediment-Dwelling Midges (*Chironomus riparius*) Under Static Conditions, Following OECD Guideline 219 Smithers Viscient Study No. 13048.6671 (Sumitomo ROW-0047) GLP, Unpublished	Y	SUM
Sayers, L.E.	2010a	S-2200 Technical Grade - Acute Toxicity To Water Fleas (*Daphnia magna*) Under Static Conditions, Following OECD Guideline #202, OPPTS Draft Guideline 850.1010, The Official Journal of the European Communities L383A, Method C.2 and JMAFF 12 Nohsan, No. 8147 Daphnia Acute Immobilization Test (2-7-2-1) and JMAFF 13 SeiSan No. 3986 Springborn Smithers Laboratories Study No. 13048.6638 (Sumitomo ROW-0013) GLP, Unpublished	Y	SUM
Sayers, L.E.	2010b	S-2200 Technical Grade - Full Life Cycle Toxicity Test with Water Fleas (*Daphnia magna*) Under Static Renewal Conditions, Following OPPTS Draft Guideline 850.1300, OECD Guideline #211, The Official Journal of the European Communities L225,	Y	SUM

Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N-R/NR	Owner
		Method C.20 JMAFF 12 NohSan, No. 8147 Daphnia spp. Reproduction Toxicity Studies (2-7-2-3) and JMAFF 13 SeiSan No. 3986 Springborn Smithers Laboratories Study No. 13048.6639 (Sumitomo ROW-0020) GLP, Unpublished		
Softcheck, K.A.	2012b	S-2167 (R-Isomer of S-2200) - 72-Hour Toxicity Test with the Freshwater Green Alga, *Pseudokirchneriella subcapitata,* Following the Official Journal of the European Communities L383A, Method C.3 Smithers Viscient Study No. 13048.6703 (Sumitomo ROW-0050) GLP, Unpublished	Y	SUM
Softcheck, K.A.	2012c	S-2354 (S-Isomer of S-2200) - 72-Hour Acute Toxicity Test with Freshwater Green Alga, *Pseudokirchneriella subcapitata,* Following the Official Journal of the European Communities L383A, Method C.3 Smithers Viscient Study No. 13048.6705 (Sumitomo ROW-0051) GLP, Unpublished	Y	SUM
Thomas, S.T., Kendall, T.Z. & Gallagher, S.P.	2012a	S-2200: A 96-hour flow-through acute toxicity test with the Sheepshead minnow (*Cyprinodon variegatus*) Wildlife International, Ltd., Report No. 263A-137A (Sumitomo ROW-0060) GLP, Unpublished	Y	SUM
Thomas, S.T., Kendall, T.Z. & Krueger, H.O.	2012	S-2200: A 96-hour flow-through acute toxicity test with the saltwater mysid (*Americamysis bahia*) Wildlife International, Ltd., Report No. 263A-138 (Sumitomo ROW-0062) GLP, Unpublished	Y	SUM
Thomas, S.T., Kendall, T.Z. & Gallagher, S.P.	2012b	S-2200: A 96-hour shell deposition test with the eastern oyster (*Crassostrea virginica*) Wildlife International, Ltd., Report No. 263A-136 (Sumitomo ROW-0071) GLP, Unpublished	Y	SUM
Thomas, S.T., Martin, K.H. & Gallagher, S.P.	2013a	S-2200: A life cycle toxicity test with the marine amphipod (*Leptocheirus plumulosus*) using spiked sediment Wildlife International, Ltd., Report No. 263A-142 (Sumitomo ROW-0073) GLP, Unpublished	Y	SUM
Thomas, S.T., Martin, K.H. & Gallagher, S.P.	2013c	S-2200: A life cycle toxicity test with the freshwater amphipod (*Hyalella azteca*) using spiked sediment Wildlife International, Ltd., Report No. 263A-141 (Sumitomo ROW-0078) GLP, Unpublished	Y	SUM

8 ANNEXES

Appendix 1: Position papers

Two position papers were submitted by the notifier and are included in full in this Appendix.

1.) The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action (Yamada, T.; 2012a)

2.) Up dated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study (Yamada., T.; 2013)

ROT-0070

Document Title

The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action

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October 10, 2012

Title: **The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action**

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CONTENTS

Page

I. Summary

S-2200 Technical Grade (abbreviated as S-2200TG in this document) is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. The general toxicity of S-2200TG has been studied in experimental animals including rats, mice, and dogs in standard bioassays. These studies have revealed that the main toxicologic target organ of S-2200TG is the liver in all species examined. In addition, thyroid was also a target organ in the rat but not in the mouse and dog. The liver finding was mainly liver hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the thyroid finding was thyroid follicular-cell hypertrophy. However, no tumourigenic findings were observed in rat or mouse carcinogenicity studies. In this document, the toxicological significance of the liver and thyroid hypertrophy caused by S-2200TG is discussed based on experimental data of S-2200TG and published information.

In this assessment, we firstly conclude that S-2200TG is a hepatic enzyme inducer via at least constitutive androstane receptor (CAR) activation in rat, similar to phenobarbital (PB), evidenced by induction of CYP2B activity and UDP-glucuronosyltransferase activity toward thyroxine (T4) (T4-UGT), and proliferation of smooth endoplasmic reticulum (SER). Therefore, the liver hypertrophy (i.e., increased liver weight and/or hepatocellular hypertrophy) caused by S-2200TG is judged to be an adaptive response by enzyme induction at least via CAR and not adverse. This activity also appears to be plausible in mouse and dog. Furthermore, this activity would theoretically operate in humans, as demonstrated by CYP2B inducers. S-2200TG at a much higher dose level induced adverse effects on liver as some additional functional changes or additional pathological findings to the hypertrophy were observed. However, the adverse effects occurred in a dose related manner and there was a threshold at relatively high exposure level; and most importantly, S-2200TG did not induce liver tumours in rat and mouse, reducing concern in human risk assessment.

Secondly, we obtained data indicating that S-2200TG increased T4-UGT and indirectly perturbed the hypothalamus-pituitary-thyroid hormone axis, and then increased thyroid follicular-cell hypertrophy in rats, which is also similar to PB, a CAR activator. The relevance of the rat thyroid abnormality to human health was assessed by using the 2006 IPCS Human Relevance Framework. The postulated mode of action (MOA) for possible induction of thyroid follicular-cell hypertrophy in rats was tested against the Bradford Hill criteria, and was found to satisfy the conditions of dose and temporal concordance, biological plausibility, coherence, strength, consistency, and specificity that fit with a well-established MOA for thyroid follicular-cell hypertrophy. Although the postulated MOA could theoretically operate in humans, marked quantitative differences in the inherent susceptibility for thyroid abnormality, especially tumour induction, to thyroid hormone imbalance in rats is not relevant to humans.

Therefore, even though the liver and thyroid hypertrophy were induced by S-2200TG treatment in experimental animals, the findings from a MOA analysis allow for the conclusion that S-2200TG does not pose a hazard to humans.

II. Introduction

S-2200TG is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. The general toxicity of S-2200TG has been studied in experimental animals including rats, mice, and dogs in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by the European Community (EC), Organisation for Economic Co-operation and Development (OECD), US. Environmental Protection Agency (US.EPA), and Ministry of Agriculture, Forestry and Fisheries of Japan (Japan MAFF). These studies have revealed that the main toxicologic target organ of S-2200TG is liver in all species examined. In addition, thyroid was also a target organ in the rat but not in mouse and dog. The liver finding was mainly liver hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the thyroid finding was thyroid follicular-cell hypertrophy. However, no tumourigenic findings were observed in rat or mouse carcinogenicity studies.

In this document, the toxicological relevance of the liver and thyroid hypertrophy caused by S-2200TG is discussed based on experimental data of S-2200TG and published information.

III. General information on liver hypertrophy

"Hepatocellular hypertrophy" is reviewed as below in "JMPR Summary Report, 5. Guidance on the interpretation of hepatocellular hypertrophy" (JMPR, 2006).

> Hepatocellular hypertrophy is a general increase in the size of the liver because of cell enlargement and accumulation of fluids. It is not attributable to tumour formation or to an increase in the number of cells (hyperplasia). An indication that hypertrophy is occurring in hepatocytes is usually an increase in the size and weight of the liver. At the cellular level, the response is a [involves] proliferation of the smooth endoplasmic reticulum (SER) that would be evident microscopically at an early stage at the tissue level as an increase in acidophilia (e.g. eosinophilia). Proliferation of SER would be confirmed by electron microscopy. Hepatocellular hypertrophy is typically related to increased functional capacity. To maintain homeostasis in the whole organism, the hepatocyte frequently responds to xenobiotic exposure by increasing its metabolic capacity via induction of xenobiotic metabolizing enzymes. Such hepatic adaptive responses usually result from chemical interaction with cellular regulatory pathways (often receptor-mediated), leading to changes in gene expression and protein synthesis, and eventually cell growth and alteration of microsomal enzyme activities. Adaptive responses are potentially beneficial in that they enhance the capacity of the organism to respond to chemical-induced stress, and are reversible. However, there are limits to these homeostatic responses and it is important to recognize when these limits have been exceeded. Because toxicity is an exposure-related phenomenon, there are lower exposures that produce effects within the control of homeostatic mechanisms and higher exposures that result in effects that exceed the capacity of these mechanisms to return the organism to its previous condition once exposure has ceased.
>
> No single effect is generally sufficient to support a determination that liver hypertrophy is adaptive or adverse. Determination of hepatotoxicity involves a detailed consideration of clinical chemistry and histopathology (or other relevant information such as histochemistry, morphometry and electron microscopy). The type, severity or magnitude, and dose–response relationship of observed effects, as well as the progression of observed lesions with duration of dosing, should be considered. It is important to evaluate whether the observed effects present a biologically plausible and consistent pattern of changes in clinical chemistry and histopathology indicative of hepatotoxicity. Sustained effects should be given more weight than transient effects.

Furthermore, we should recognize that sustained treatment with enzyme inducers in rodents for a long time often (but not always) induce liver tumours; e.g., activators via constitutive androstane receptor (CAR) and peroxisome proliferators activated receptor alpha (PPARα) have been well studied (Cohen and Arnold, 2011; Klaunig et al., 2003; Osimitz and Lake, 2009; Yamada et al.,

2009). The nuclear receptor CAR mediates the response evoked by a class of xenobiotics known as the `phenobarbital-like inducers' (Wei et al., 2000).

IV. General information on thyroid hypertrophy

There is an excellent review of thyroid biology (Dellarco, et al., 2006). Current understanding about thyroid related-alterations is summarized below:

> There are considerable data from studies in laboratory rodents demonstrating the relationship between sustained perturbation of the hypothalamic–pituitary–thyroid axis, prolonged stimulation of the thyroid gland by thyroid stimulating hormone (TSH), and the progression of thyroid follicular-cells to hypertrophy, hyperplasia, and eventually neoplasia (Hard, 1998; Hurley et al., 1998; IARC, 2001; McClain, 1995). Increased secretion of TSH may result via several mechanisms, including increased hepatic clearance of thyroxine (T4) by UDP-glucuronosyltransferase activity toward T4 (T4-UGT).
>
> Circulating levels of T4 are monitored by the thyrotropic cells of the pituitary gland that are responsible for the synthesis of TSH. In the pituitary gland T4 is metabolized by 5-deiodinase type II to T3, which then binds to specific receptors in the [pituitary] cell nucleus [(Capen, 1997)]. A decrease in T3 receptor occupancy results in stimulation of TSH synthesis and secretion. Studies in vivo have shown that injection of rats with TSH leads to reductions in thyroid follicular-cell nuclear statin, a non-proliferation specific nuclear antigen, indicating that these cells were leaving the nondividing state to resume the cell cycle (Bayer et al., 1992). This study showed that low, repeated doses of TSH (0.25 IU/rat twice daily) produced a cumulative response in nuclear statin levels over 10 days, and the response returned to normal resting levels within 5 days of cessation of TSH injections. Reduction in nuclear statin is also an early event that parallels the earliest known pinocytotic response to TSH. These data are consistent with increased TSH concentrations alone causing thyroid follicular-cells of rats to enter a state of preproliferation. [Hepatic UGT activity is regulated by the nuclear receptor CAR (Sugatani et al., 2001), and its stimulation leads to metabolism of T4 and consequent increased TSH and thyroid hypertrophy and proliferation.]

V. Assessment of toxicological relevance of S-2200TG-induced liver and thyroid alterations

V-1. Genotoxicity and carcinogenicity of S-2200TG

S-2200TG was not genotoxic in a battery of *in vitro* and *in vivo* assays: reverse mutation test in a bacterial system, gene mutation test in Chinese Hamster V79 cells, chromosomal abbreviation test in Chinese Hamster lung cells (CHL/IU), and micronucleus test in CD-1 mice.

The carcinogenicity of S-2200TG has been studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice.

Male and female Crl:CD1(ICR) mice were fed 0 (control), 700, 2000, or 7000 ppm S-2200TG (purity, 93.4%) in the diet for 78 weeks (average chemical intakes: 83, 239, and 824 mg/kg/day for males; 99, 280, and 994 mg/kg/day for females, respectively). The No Observed Adverse Effect Level (NOAEL) for this study was considered to be 7000 ppm (824 mg/kg/day for males and 994 mg/kg/day for females) following 78 weeks of treatment since no increased tumourigenicity occurred. Male and female Crl:WI(Han) rats were fed 0 (control), 400, 2000, 7000, or 15000 ppm S-2200TG (purity, 93.4%) in the diet for 104 weeks (average chemical intakes: 21, 105, 376, and 804 mg/kg/day for males; 27, 135, 475 and 1016 mg/kg/day for females, respectively). Body weight suppressions were observed at 15000 ppm in males and 2000 ppm and higher in females. The NOAEL for this study was considered to be 2000 ppm (105 mg/kg/day) for males and 400 ppm (26.7 mg/kg/day) for females following 104 weeks of treatment.

No statistically-significant increase of neoplastic findings was observed in any organs of treated animals in both studies. Therefore, S-2200TG is concluded not to be carcinogenic.

V-2. Evidence for enzyme induction by S-2200TG

Based on experimental data described below, S-2200TG is a hepatic enzyme inducer via at least CAR activation in rat and mouse, similar to phenobarbital (PB).

A. Rat

As an analysis for mode of action (MOA) for S-2200TG-induced liver and thyroid alterations in rats, a study was conducted to evaluate the dose response, time course, and reversibility of alterations at an early phase of treatment with S-2200TG, mainly focusing on liver enzyme induction, hepatocellular replicative DNA synthesis, and thyroid hormone levels and morphology. Details are presented in the Technical Report (Asano, 2012). Male and female Crlj:WI rats were fed diets containing 0 (control), 400, 2000, 7000 and 15000 ppm S-2200TG for 7 days. These dose levels are identical to those in the 2-year bioassay. To evaluate time course of alterations at 15000 ppm, male and female Crlj:WI rats were also fed diets containing 0 and 15000 ppm for 14 days, and data from both 7 and 14-day treatment groups were compared to determine whether enhancement or attenuation of alterations due to longer treatment was observed. Furthermore, to evaluate reversibility, male and female Crlj:WI rats were fed diets containing 0 and 15000 ppm S-2200TG for 7 days followed by 7-day cessation of the treatment. As a positive control for a CAR activator, 1000 ppm PB groups were included in each phase of the study.

Under conditions of the present study, treatment with S-2200TG caused no deaths, did not show severe toxicity or marked abnormalities in clinical signs, body weight or food consumption that could confound evaluation of the main target endpoints.

In rats treated with S-2200TG for 7 days, hepatic alterations were observed in a dose-related manner; they included increases of liver weight, enlarged liver, diffuse hepatocellular hypertrophy, CYP2B and T4-UGT activities, and replicative DNA synthesis of hepatocytes (determined by 5-bromo-2'-deoxyuridine (BrdU) labeling index). In general, clear effects were observed at 7000 ppm and higher in both sexes. However, CYP4A activity revealed no remarkable increase. Electron microscopic examination revealed SER was increased, but peroxisome was not changed in the hepatocytes from the animals administered S-2200TG at 15000 ppm. These findings are summarized in Tables 1 and 2.

Table 1. Summary findings from 7-day treatment study in rats (Male)

Endpoints		7-day Treatment group					
			S-2200TG				PB
		Control	400ppm	2000ppm	7000ppm	15000ppm	1000ppm
Test Item Intake (mg/kg/day)		0	23.3	115.7	378.9	744.4	57
Death #		0/10	0/10	0/10	0/10	0/10	0/10
Final Body Weight (g)		407±23.3	394±17.4	394±10.1	388±14.8*	386±31.9	398±29.7
Body Weight Gain (g)		38±5.0	34±6.5	34±4.9	28±5.6**	27±12	35±12
Food Consumption (g/animal/day)		23±0.7	22±0.5	22±0.8	21±0.8*	21±1.8*	21±1.1*
Liver Weight	Absolute (g)	14.86±1.381	14.26±1.07	14.83±1.005	15.6±0.835	17.52±2.173*	17.95±1.643**
	(vs. control, fold)	1	0.96	1.00	1.05	1.18	1.21
	Relative (g/body weight)	3.65±0.22	3.61±0.177	3.77±0.214	4.03±0.215**	4.53±0.342**	4.51±0.278**
	(vs. control, fold)	1	0.99	1.03	1.10	1.24	1.24
Thyroid Weight	Absolute (g)	21±3	22±3.4	23±6.3	21±3.8	24±3.3	25±5.7
	(vs. control, fold)	1	1.05	1.10	1.00	1.14	1.19
	Relative (g/body weight)	5.2±0.74	5.6±0.98	5.8±1.58	5.4±0.88	6.2±0.92	6.2±1.43
	(vs. control, fold)	1	1.08	1.12	1.04	1.19	1.19
BrdU labeling index of hepatocytes (%)		0.94±0.47	0.92±0.52	1.71±1.01	2.19±0.92**	3.66±2.47**	3.65±1.87**
	(vs. control, fold)	1	0.98	1.82	2.33	3.89	3.88
Hepatic CYP2B activity	(pmol/min/mg S9 protein)	32±15.3	47±10.1	92±9.7*	278±94.3*	433±119.6*	800±263.7*
	(vs. control, fold)	1	1.47	2.88	8.69	13.53	25.00
Hepatic CYP4A activity	(pmol/min/mg S9 protein)	125±19.1	122±24.4	124±16.5	151±13.7	155±24.6	209±24.5**
	(vs. control, fold)	1	0.98	0.99	1.21	1.24	1.67
Hepatic T4-UGT	(pmol/min/mg S9 protein)	0.41±0.054	0.51±0.024*	0.54±0.075	0.62±0.051*	0.61±0.046*	0.79±0.122*
	(vs. control, fold)	1	1.24	1.32	1.51	1.49	1.93
Serum TSH	(ng/mL)	8.6±2.76	7.3±2.33	7.5±2.5	6.7±2.12	7.5±2.96	10.6±3.42
	(vs. control, fold)	1	0.85	0.87	0.78	0.87	1.23
Serum T3	(μg/dL)	0.5±0.07	0.5±0.1	0.5±0.08	0.4±0.08	0.5±0.1	0.5±0.11
	(vs. control, fold)	1	1.00	1.00	0.80	1.00	1.00
Serum T4	(μg/dL)	5.04±0.722	5.35±0.848	5.36±0.71	4.56±0.897	4.09±0.869*	4.45±1.305
	(vs. control, fold)	1	1.06	1.06	0.90	0.81	0.88
Gross pathology #	Liver						
	Enlarged	0/10	0/10	0/10	0/10	3/10	0/10
Histopathology #	Liver						
	Hypertrophy, hepatocyte, centrilobular	0/10	0/10	0/10	0/10	0/10	10/10**
	Hypertrophy, hepatocyte, diffuse	0/10	0/10	1/10	8/10**	9/10**	0/10
	Thyroid						
	Hypertrophy, follicular, diffuse	1/10	1/10	2/10	2/10	3/10	5/10
Histopathology #	Liver						
electron microscopy	Proliferation, SER	0/2	ND	ND	ND	1/2	ND

Data presents mean±SD, n=6-10. #: Data is scored as number of animals exhibiting findings out of total number of animals examined.
*: p<0.05, **: p<0.01. Red shadow presents biologically significant change. ND: Not Determined

Table 2. Summary findings from 7-day treatment study in rats (Female)

Endpoints		7-day treatment group					
			S-2200TG				PB
		Control	400ppm	2000ppm	7000ppm	15000ppm	1000ppm
Test Item Intake (mg/kg/day)		0	25.7	131.2	420.2	811.8	66.2
Death #		0/10	0/10	0/10	0/10	0/10	0/10
Final Body Weight (g)		271±17.5	266±10.7	266±12.2	263±8.8	255±17.6	269±16.4
Body Weight Gain (g)		24±11.6	23±6.9	23±8.9	21±9.2	12±5.1**	19±12.7
Food Consumption (g/animal/day)		17±0.5	17±1.1	17±1.1	16±0.5	15±1.1	16±0.9
Liver Weight	Absolute (g)	9.3±0.797	9.08±0.761	9.64±0.458	9.99±0.544	10.67±0.943**	10.96±1.197**
	(vs. control, fold)	1	0.98	1.04	1.07	1.15	1.18
	Relative (g/body weight)	3.44±0.125	3.41±0.241	3.63±0.12*	3.79±0.095**	4.17±0.118**	4.07±0.232**
	(vs. control, fold)	1	0.99	1.06	1.10	1.21	1.18
Thyroid Weight	Absolute (g)	18±4	18±2.8	18±3.1	23±3.1**	22±3.5*	21±3.4
	(vs. control, fold)	1	1.00	1.00	1.28	1.22	1.17
	Relative (g/body weight)	6.5±1.38	6.9±1.09	6.7±1.17	8.6±1.12**	8.5±1.38**	7.9±1.04*
	(vs. control, fold)	1	1.06	1.03	1.32	1.31	1.22
BrdU labeling index of hepatocytes (%)		0.99±0.63	1.90±0.71	1.95±1.06	2.23±0.96*	2.39±1.55**	3.05±1.10**
	(vs. control, fold)	1	1.92	1.97	2.25	2.41	3.08
Hepatic CYP2B activity	(pmol/min/mg S9 protein)	1.5±1.05	1.7±0.77	4.5±2.15*	38.1±15.39*	117.9±70.55*	397.8±79.35*
	(vs. control, fold)	1	1.13	3	25.4	78.6	265.2
Hepatic CYP4A activity	(pmol/min/mg S9 protein)	177±29.2	167±41.3	152±35.5	158±31.3	150±36.1	229±16.9*
	(vs. control, fold)	1	0.94	0.86	0.89	0.85	1.29
Hepatic T4-UGT	(pmol/min/mg S9 protein)	0.46±0.069	0.43±0.033	0.46±0.071	0.53±0.057	0.62±0.074**	0.56±0.06*
	(vs. control, fold)	1	0.93	1	1.15	1.35	1.22
Serum TSH	(ng/mL)	6.1±1.71	6.3±2.13	6.7±2.14	9.1±4.99	15.1±6.22**	10.2±3.8**
	(vs. control, fold)	1	1.03	1.10	1.49	2.48	1.67
Serum T3	(μg/dL)	0.6±0.19	0.6±0.12	0.6±0.21	0.6±0.15	0.6±0.08	0.6±0.14
	(vs. control, fold)	1	1.00	1.00	1.00	1.00	1.00
Serum T4	(μg/dL)	4.7±1.412	4.7±1.124	4.52±1.331	4.34±1.24	3.95±1.311	3.17±0.641**
	(vs. control, fold)	1	1.00	0.96	0.92	0.84	0.67
Gross pathology #	Liver						
	Enlarged	0/10	0/10	0/10	0/10	0/10	5/10*
Histopathology #	Liver						
	Hypertrophy, hepatocyte, centrilobular	0/10	0/10	0/10	0/10	0/10	10/10**
	Hypertrophy, hepatocyte, diffuse	1/10	1/10	5/10	6/10*	8/10**	0/10
	Thyroid						
	Hypertrophy, follicular, diffuse	0/10	1/10	1/10	4/10*	6/10**	3/10
Histopathology #	Liver						
electron microscopy	Proliferation, SER	0/2	ND	ND	ND	1/2	ND
	Enlargement, lipid droplet	0/2	ND	ND	ND	1/2	ND

Data present mean±SD, n=6-10. #: Data is scored as number of animals exhibiting findings out of total number of animals examined.
*: p<0.05, **: p<0.01. Red shadow presents biologically significant change. ND: Not Determined

Figure 1 shows that CYP2B and T4-UGT activities were increased in both sexes. These increases were less than that induced by PB 1000 ppm except for T4-UGT in females.

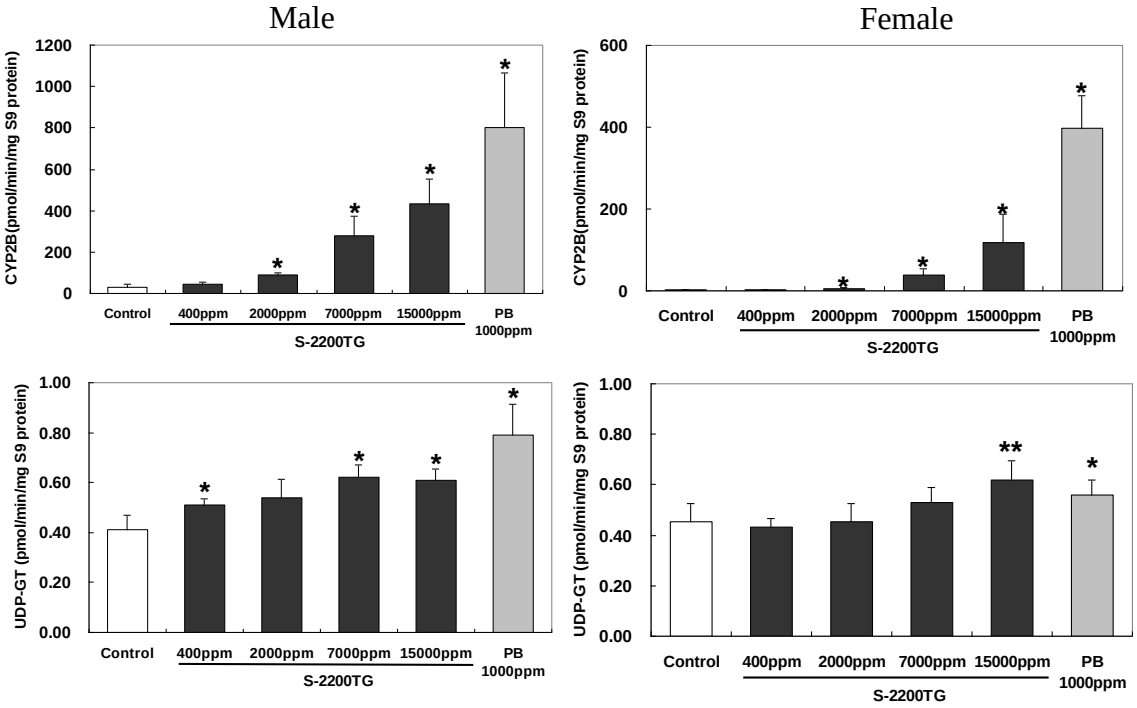

Figure 1. Hepatic CYP2B and T4-UGT induction in rats treated with S-2200TG or phenobarbital (PB) for 7 days. Data represent mean ± SD, N=6. * p<0.05, **p<0.01.

Compared to those observed after 7-day treatment, 14-day treatment revealed equivalent or slightly enhanced alterations of liver weight and morphology. For replicative DNA synthesis, continuous treatment with S-2200TG 15000 ppm for 14 days attenuated the increase of BrdU labeling indices, indicating a transient increase of rate of replicative DNA synthesis (Figure 2). These findings were consistent with those caused by PB.

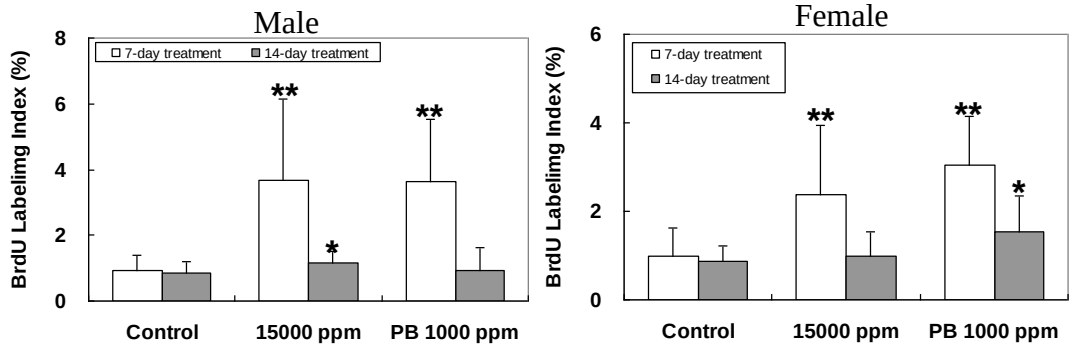

Figure 2. Hepatic replicative DNA synthesis determined by BrdU labeling indices in rats treated with S-2200TG or phenobarbital (PB) for 7 and 14 days. Data represent mean ± SD, N=10. *p<0.05, **p<0.01.

In rats treated with S-2200TG for 7 days, slight decrease of serum T4 levels, slight increase of TSH, and slight increase of thyroid weight with diffuse follicular-cell hypertrophy were observed in females in a dose-related manner irrespective of statistical significance (Tables 1 and 2). These effects were not recognized in males although serum T4 was significantly decreased (Tables 1 and 2). However, after 14-day treatment with S-2200TG, these findings were more clearly observed in both sexes (Figure 3). Taken together with induction of T4-UGT, these findings suggest that the decrease of T4 occurred prior to the increase of TSH and thyroid hypertrophy.

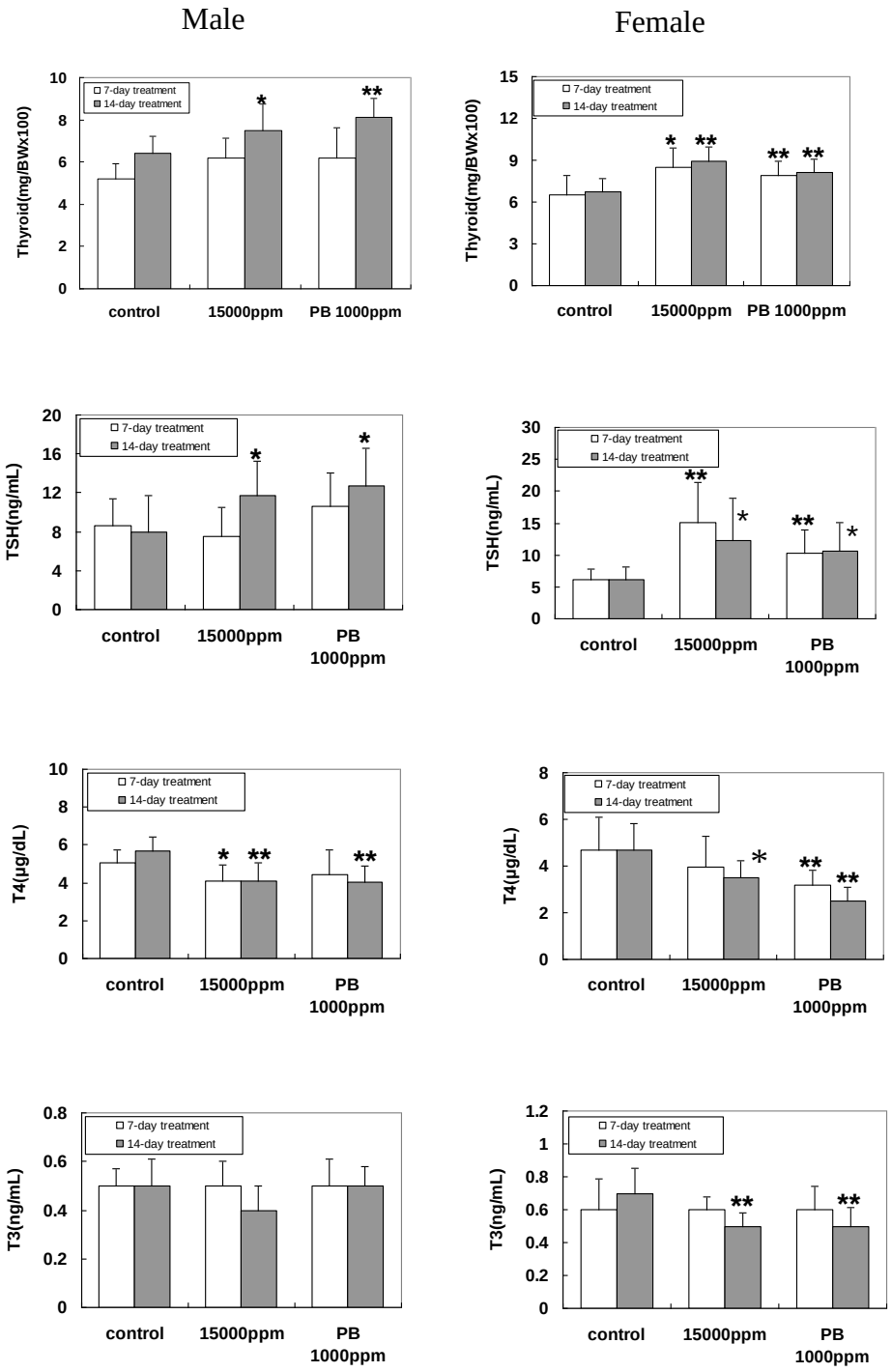

Figure 3. Comparison of alterations on relative thyroid weight and serum hormone levels in rats treated with S-2200TG or phenobarbital (PB) for 7 or 14 days. Data represent mean ± SD, N=10. * p<0.05, ** p<0.01.

Regarding reversibility, all events including increased liver weights, increased enzyme induction, hepatocellular hypertrophy, decreased T4, increased TSH, increased thyroid weight, and thyroid follicular-cell hypertrophy were attenuated after 7-day cessation of S-2200TG treatment, suggesting that the alterations of rat liver and thyroid by S-2200TG are reversible similar to what occurs with PB.

In conclusion, the present study demonstrated that treatment with S-2200TG in the rat revealed increased liver weight with diffuse hepatocellular hypertrophy and proliferation of SER, remarkable increase of CYP2B activity, and transient increase of rate of replicative DNA synthesis, with a dose response and reversibility. Furthermore, S-2200TG increased T4-UGT activity and secondarily perturbed the hypothalamus-pituitary-thyroid hormone axis. These effects are similar to PB, a CAR activator. Therefore, it is reasonable to conclude that S-2200TG is a hepatic enzyme inducer via at least CAR activation in rat, similar to PB.

B. Mouse

The study was conducted to evaluate whether S-2200TG induces hepatic metabolic enzymes via CAR activation in mouse liver (Yamada, 2012). Male Crlj:CD1(ICR) mice were fed diets containing 0 (control) or 7000 ppm S-2200TG for 7 days, and then hepatic CYP2B activity (determined by 7-pentoxyresorufin O-depentylase activity) was examined. 7000 ppm is the highest dose in the mouse 1.5-year bioassay. In addition, replicative DNA synthesis of hepatocytes (determined by BrdU labeling index) was also examined because CAR activators often increase BrdU labeling index of hepatocytes at an early phase of treatment (Jones et al., 2009). Under conditions of the present study, treatment with S-2200TG caused no deaths, did not show severe toxicity, that might confound evaluation of hepatic CYP2B induction or BrdU labelling index, and no major clinical signs, body weight or food consumption changes occurred. The findings are summarized in Table 3.

Absolute liver weight tended to increase but not statistically significantly (1.06-fold of control value), whereas relative liver weight was significantly increased by S-2200TG treatment (1.07-fold of control value). No clear alterations were observed in liver gross pathology. By histopathology, slight eosinophilic change/hypertrophy of hepatocytes was observed in three of ten animals treated with S-2200TG 7000 ppm, none in controls. No necrosis was observed. Hepatic CYP2B activity was also significantly increased by S-2200TG treatment (1.71-fold of control value). No clear alterations were observed in replicative DNA synthesis of hepatocytes.

In conclusion, the present study demonstrated that treatment with S-2200TG in mouse revealed an increase of liver weight with slight eosinophilic change/hypertrophy of hepatocyte and increase of CYP2B activity, but much less than with PB (unpublished data). Therefore, it is reasonable to conclude that S-2200TG is a weak hepatic enzyme inducer via at least CAR activation in mouse. However, in contrast to PB (Jones, et. al, 2009), S-2200TG at this dose level tested did not significantly enhance replicative DNA synthesis of hepatocytes at this early phase of treatment.

Table 3. Summary findings from 7-day treatment study in mice

Endpoints		Groups	
		Control	S-2200TG 7000 ppm
Test Item Intake (mg/kg/day)		0	814
Death #		0/10	0/10
Final Body Weight (g)		43.8 ± 1.89	43.3 ± 2.26
Total Body Weight Gain (g)		2.8 ± 0.88	2.3 ± 0.84
Food Consumption at Day 6 (g/animal/day)		5.2 ± 0.48	5.2 ± 0.36
Liver Weight	Absolute (g)	2.50 ± 0.193	2.65 ± 0.216
	(Fold change compared to control)	1	1.06
	Relative (g/body weight)	5.72 ± 0.356	6.12 ± 0.315*
	(Fold change compared to control)	1	1.07
S9 ptotein (mg/g liver)		167 ± 19.2	192 ± 17.2*
	(Fold change compared to control)	1	1.15
Hepatic CYP2B activity (pmol/min/mg S9 protein)		35 ± 4.2	60 ± 8.6**
	(Fold change compared to control)	1	1.71
BrdU labeling index of hepatocytes (%)		3.59 ± 1.93	3.89 ± 1.97
	(Fold change compared to control)	1	1.08
Gross pathology #	Liver		
	No remarkable findings	10/10	10/10
Histopathology #	Liver		
	No remarkable findings	10/10	7/10
	Eosinophilic change/hypertrophy, hepatocyte	0/10	3/10
	Brownish pigment, hepatocyte, focal	0/10	2/10
	Cell infiltration, mononuclear cell, focal	2/10	4/10
	Necrosis, focal	2/10	3/10

Data presents mean±SD, N=6-10. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *p<0.05, **p<0.01. Red shadow presents biologically significant change.

V-3. Assessment for toxicological relevance of liver hypertrophy observed in general toxicity and/or carcinogenicity studies with S-2200TG

As mentioned above, several toxicology studies have revealed that the main toxicologic target organ of S-2200TG is liver in all species examined. The liver finding was mainly liver hypertrophy (increased liver weight and/or hepatocellular hypertrophy). In addition, thyroid was also a target organ in the rat but not in mouse and dog. The thyroid finding was thyroid follicular-cell hypertrophy. Toxicological relevance of the liver findings were first assessed in line mainly with the guidance in the JMPR Summary Report (JMPR, 2006), and then the toxicological relevance of the thyroid findings were assessed.

A. Rat

The findings in rat studies are summarized in Tables 4 and 5. The general toxicity and carcinogenicity studies of S-2200TG in rats revealed liver hypertrophy (i.e., increased liver weight and/or hepatocellular hypertrophy) and thyroid hyperplasia (weight change of thyroid was not examined but thyroid follicular-cell hypertrophy was observed by histopathological examination). Apart from the increased liver weight and hepatocellular hypertrophy, γ-glutamyltranspeptidase and total cholesterol in peripheral blood were increased at some dose levels. However, no changes were observed in alanine aminotransferase and aspartate aminotransferase at any doses examined. Histopathologically, other findings, including cytotoxicity and necrosis, were not observed in all phases examined. Only hepatocellular vacuolation was increased at 7000 and 15000 ppm after 2-year treatment in both sexes. Furthermore, neoplastic and pre-neoplastic findings were not observed.

Regarding the liver hypertrophy caused by S-2200TG, since induction of microsome enzyme by S-2200TG is already evident as mentioned above (Asano, 2012; Yamada, 2012), it could be an adaptive response when neither concomitant abnormality of several endpoints in blood

biochemistry or other pathological findings were observed. Therefore, in the 90-day study, the Lowest Adverse Effect Level (LOAEL) on the liver was concluded to be at 20000 ppm of both sexes because significant changes of two parameters such as γ-glutamyltranspeptidase and total cholesterol accompanied the liver hypertrophy. However, at 10000 ppm of both sexes, only serum total cholesterol was slightly increased. Therefore, the findings at 10000 ppm are considered an adaptive response but not adverse.

After 1-year treatment (satellite group in the combined chronic and carcinogenicity study), similar changes to 20000 ppm in the 90-day study were observed at 15000 ppm but not at 7000 ppm. Therefore, the LOAEL in the liver after 1-year treatment is 7000 ppm in both sexes. After 2-year treatment, the liver hypertrophy was observed at 7000 and 15000 ppm in males and 2000, 7000, and 15000 ppm in females. Although blood biochemistry was not examined in this carcinogenicity study, increased hepatocellular vacuolation was also observed in addition to hypertrophy at 7000 and 15000 ppm. Thus, the LOAEL for the liver after 2-year treatment is considered to be 7000 ppm in both sexes.

Table 4. Summary of liver and thyroid findings in male rats

Endpoints	Dose levels (ppm)								
	0	400	800	2000	4000	7000	10000	15000	20000
90-Day Study									
Chemical intake (mg/kg/day)	0	na	54	na	282.6	na	742.7	na	1544.6
Final body weight	100	na	103	na	99	na	96	na	92
Absolute liver weight	100	na	108	na	**114****	na	**122****	na	**144****
Relative liver weight	100	na	105	na	**115****	na	**128****	na	**157****
Hepatocellular hypertrophy #	0/12	na	0/12	na	**12/12**	na	**12/12**	na	**12/12**
γ-Glutamyltranspeptidase	2	na	2	na	2	na	2	na	**5****
Total cholesterol	100	na	110	na	115	na	**130****	na	**140****
Thyroid follicular-cell hypertrophy #	2/12	na	2/12	na	**6/12**	na	**9/12***	na	**7/12**
1-Year Study									
Chemical intake (mg/kg/day)	0.0	25.5	na	130.3	na	448.8	na	991.8	na
Final body weight	100	109*	na	99	na	96	na	92	na
Absolute liver weight	100	**117****	na	109	na	**112***	na	**127****	na
Relative liver weight	100	107	na	**110****	na	**117****	na	**140****	na
Hepatocellular eosinophilia / hypertrophy #	0/19	0/19	na	0/19	na	**15/20****	na	**20/20****	na
γ-Glutamyltranspeptidase	2	2	na	2	na	2	na	**8****	na
Total cholesterol	100	100	na	104	na	117	na	**126***	na
Thyroid follicular-cell hypertrophy #	1/19	0/19	na	1/19	na	**9/20****	na	**18/20****	na
2-Year Study									
Chemical intake (mg/kg/day)	0.0	21.0	na	105.1	na	375.6	na	804.3	na
Final body weight	100	101	na	94	na	96	na	**89****	na

Absolute liver weight	100	103	na	97	na	114	na	103	na
Relative liver weight	100	99	na	100	na	108	na	**114***	na
Hepatocellular eosinophilia / hypertrophy #	7/50	11/50	na	13/50	na	**30/50****	na	**37/50****	na
Hepatocellular vacuolation #	28/50	39/50*	na	37/50	na	**38/50$**	na	**45/50****	na
Eosinophilic focus #	0/50	0/50	na	1/50	na	1/50	na	0/50	na
Hepatocellular adenoma #	1/50	0/50	na	0/50	na	0/50	na	2/50	na
Thyroid follicular-cell hypertrophy #	1/50	2/50	na	0/50	na	0/50	na	**11/50****	na
Thyroid follicular-cell adenoma #	7/50	4/50	na	7/50	na	7/50	na	7/50	na
Thyroid follicular-cell carcinoma #	0/50	0/50	na	2/50	na	1/50	na	0/50	na

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: $p<0.05$, **: $p<0.01$. Shadow presents biologically significant change. $: The finding in males of 7000 ppm is considered toxicologically significant because data from terminal-kill animals indicates statistically significant increase. "na" means data not available.

Table 5. Summary of liver and thyroid findings in female rats

	Dose levels（ppm）								
	0	400	800	2000	4000	7000	10000	15000	20000
90 Day Study									
Chemical intake (mg/kg/day)	0	na	61.6	na	320.1	na	788.5	na	1886.5
Final body weight	100	na	97	na	97	na	98	na	96
Absolute liver weight	100	na	95	na	105	na	**117****	na	**144****
Relative liver weight	100	na	98	na	108	na	**119****	na	**150****
Hepatocellular hypertrophy #	0/12	na	0/12	na	**4/11***	na	**9/12****	na	**12/12****
γ-Glutamyltranspeptidase	2	na	2	na	2	na	3	na	**4****
Total cholesterol	100	na	113	na	120	na	**167****	na	**173****
Thyroid follicular-cell hypertrophy #	2/12	na	1/12	na	**4/11**	na	**5/12**	na	**6/12**
1-Year Study									
Chemical intake (mg/kg/day)	0.0	31.3	na	151.4	na	535.3	na	1138.9	na
Final body weight	100	96	na	98	na	97	na	**91***	na
Absolute liver weight	100	96	na	103	na	**117****	na	**124****	na
Relative liver weight	100	100	na	105	na	**121****	na	**135****	na
Hepatocellular eosinophilia / hypertrophy #	0/20	1/20	na	1/19	na	**17/19****	na	**15/20****	na
γ-Glutamyltranspeptidase	2	2	na	2	na	2	na	**6****	na
Total cholesterol	100	90	na	115	na	**155****	na	**150****	na
Thyroid follicular-cell hypertrophy #	0/20	0/20	na	0/19	na	**9/19****	na	**15/20****	na
2-Year Study									
Chemical intake (mg/kg/day)	0.0	26.7	na	135.2	na	475.0	na	1016.2	na
Final body weight	100	97	na	**89****	na	**89****	na	**80****	na

	Dose levels（ppm）								
	0	400	800	2000	4000	7000	10000	15000	20000
Absolute liver weight	100	104	na	105	na	103	na	111	na
Relative liver weight	100	95	na	**115***	na	**114***	na	**128****	na
Hepatocellular eosinophilia / hypertrophy #	16/50	22/50	na	**32/50****	na	**43/50****	na	**42/50****	na
Hepatocellular vacuolation #	20/50	13/50	na	19/50	na	**29/50$**	na	**38/50****	na
Eosinophilic focus	0/50	0/50	na	0/50	na	1/50	na	1/50	na
Hepatocellular adenoma	0/50	0/50	na	0/50	na	0/50	na	1/50	na
Thyroid follicular-cell hypertrophy #	0/50	1/50	na	2/50	na	0/50	na	4/50	na
Thyroid follicular-cell adenoma #	2/50	2/50	na	2/50	na	5/50	na	1/50	na
Thyroid follicular-cell carcinoma #	0/50	0/50	na	0/50	na	1/50	na	0/50	na

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: $p<0.05$, **: $p<0.01$. Shadow presents biologically significant change. $: The finding in females at 7000 ppm is considered toxicologically significant because data from terminal-kill animals indicates statistically significant increase. “na” means data not available.

To determine whether progression of liver hypertrophy with duration of treatment is observed, further analysis was conducted. As shown in Figure 4, the relative liver weight was increased by S-2200TG treatment in a dose related manner. Generally, dose response of the liver weight change adjusted by chemical intake was similar among the three studies, i.e., potency of liver hypertrophy is similar irrespective of the treatment period. These findings suggest that the stress caused by S-2200TG at dose levels tested was within the control of homeostatic mechanisms. In fact, as mentioned above, no cytotoxic finding was observed.

Regarding thyroid, details are discussed later in this document.

No increase of tumours in any organs, including liver and thyroid, was observed in rats treated with S-2200TG at 15000 ppm which is the limiting dose level in the 2 year study.

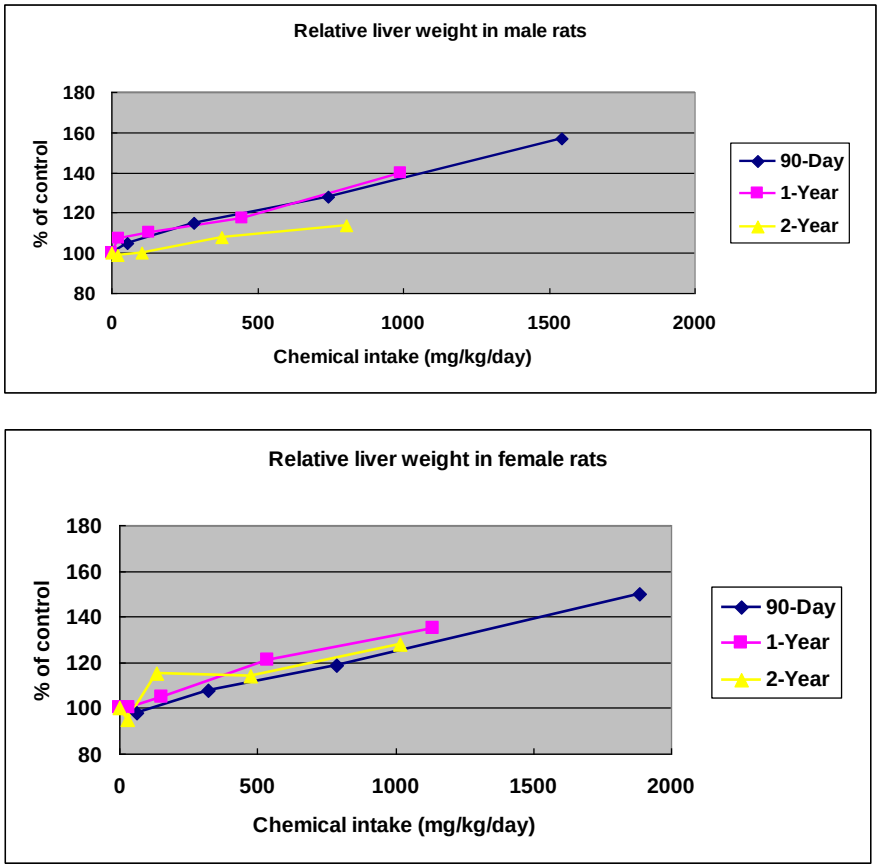

Figure 4. Relative liver weight changes in rats treated with S-2200TG for 90 days, 1 year, and 2 years. Date represent % of control values at each dose levels; 0, 800, 4000, 10000, and 20000 ppm for the 90-Day study; 0, 400, 2000, 7000, and 15000 ppm for the 1- and 2-Year studies.

B. Mouse

As shown in Table 6, S-2200TG also increased mouse liver weight in the 90-day, 1-year (satellite group in the carcinogenicity study), and 1.5-year studies, but without any associated liver pathological findings including hepatocellular hypertrophy and changes of blood biochemistry. S-2200TG is also a CYP2B inducer in mouse liver (Yamada, 2012). Therefore, these findings suggest that the increased liver weight by S-2200TG in mice is an adaptive response, but not adverse effect.

No apparent abnormality compared to control was observed in mouse thyroid.

No increase of tumours in any organs, including liver and thyroid, was observed in mice treated with S-2200TG at the limited dose level for 1.5 year.

Table 6. Summary of liver findings in mice

	Dose levels (ppm)											
	Male						Female					
	0	700	1750	2000	3500	7000	0	700	1750	2000	3500	7000
90-Day Study												
Chemical Intake (mg/kg/day)	0	na	204	na	405	807	0	na	252	na	529	1110
Final body weight	100	na	101	na	98	102	100	na	98	na	96	94
Absolute liver weight	100	na	105	na	107	**117****	100	na	110	na	104	114
Relative liver weight	100	na	104	na	**109****	**115****	100	na	**112***	na	108	**122****
1-Year Study												
Chemical Intake (mg/kg/day)	0	88.4	na	255	na	883	0	104	na	325	na	1050
Final body weight	100	99	na	95	na	95	100	100	na	102	na	89
Absolute liver weight	100	105	na	103	na	111	100	103	na	110	na	113
Relative liver weight	100	106	na	108	na	**115****	100	103	na	108	na	**125****
1.5-Year Study												
Chemical Intake (mg/kg/day)	na	82.5	na	239	na	824	na	99.2	na	280	na	994
Final body weight	100	99	na	102	na	98	100	102	na	105	na	97
Absolute liver weight	100	106	na	106	na	115	100	114	na	120	na	109
Relative liver weight	100	106	na	105	na	**115****	100	105	na	112	na	111
Hepatocellular adenoma #	7/51	4/51	na	10/51	na	6/51	0/51	0/51	na	0/51	na	0/51
Hepatocellular carcinoma #	0/51	2/51	na	1/51	na	0/51	0/51	0/51	na	0/51	na	0/51

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined.*: $p<0.05$, **: $p<0.01$. Shadow presents statistically significant change but toxicologically not significant because no concomitant other histopathology and blood biochemistry. "na" means data not available.

C. Dog

In the dog 90-day study, S-2200TG also increased liver weight (in males, 12000 and 40000 ppm; and in females, 4000 ppm and above) (Table 7). Histopathologically, centrilobular hepatocellular swelling was present in males administered 12000 and 40000 ppm and in females administered 4000 and 12000 ppm. Females at 40000 ppm did not show this finding. Reason for lack of this finding is unknown, but severe suppression of body weight may be related. [Notifier considers that "centrilobular hepatocellular swelling" means "centrilobular hepatocellular hypertrophy" because "centrilobular hepatocellular hypertrophy" was recorded in the 1-year dog study.] We do not have direct evidence for hepatic enzyme induction by S-2200TG in dog liver. However, we know that S-2200TG induces at least CYP2B accompanied with increased liver weight and hepatocellular hypertrophy in rat and mouse liver. These finding suggests that S-2200TG is also likely to induce hepatic enzyme in dogs.

Some additional associated changes in blood biochemistry and other pathological findings were

observed at 12000 ppm and higher in both sexes. The alterations of blood biochemistry were increases in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, triglyceride, and globulin; and decreases in total cholesterol, albumin, albumin/globulin ratios and glucose. In addition, other abnormalities were also observed; pigment and centrilobular degeneration, periportal/centrilobular fibrosis, etc. However, no additional findings were observed in females at 4000 ppm. Therefore, the increased liver weight and hepatocellular swelling (hypertrophy) at 4000 ppm in females are not adverse but adaptive responses. Based on these findings, the LOAEL in the liver in the 90-day dog study was determined to be 12000 ppm in both sexes.

As shown in Table 8, in the dog 1-year study, S-2200TG also increased liver weight (males, 4000 and 8000 ppm; and females, 8000 ppm). S-2200TG-related disturbances in blood biochemistry parameters includes increases in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyltransferase (males, 4000 and/or 8000 ppm; females, 8000 ppm), and decreases in albumin and total cholesterol (females, 8000 ppm). Histopathologically, liver hepatocyte hypertrophy was recorded for males and females administered 4000 and 8000 ppm. Increased levels of hepatocyte pigment (males, 4000 and 8000 ppm; and females, 8000 ppm) together with a marginal increase in pigmented macrophages (both sexes, 8000 ppm), centrilobular degeneration (one male and one female administered 8000 ppm), and portal fibrosis/bile duct proliferation (one male administered 8000 ppm) were observed. Overall, since S-2200TG induced liver hypertrophy with concomitant biochemical changes and other histopathological findings, the LOAEL in the liver in the dog 1-year study was determined to be 4000 ppm in males and 8000 ppm in females.

No apparent abnormality was observed in dog thyroid.

Table 7. Summary of liver findings in the dog 90-day study

Endpoints	Examination (Week)	Dose levels (ppm)							
		Male				Female			
		0	4000	12000	40000	0	4000	12000	40000
Chemical intake (mg/kg/day)		0	90.9	267.8	933.1	0	102.7	304.4	820.4
Final body weight		100	96	97	**79***	100	96	96	**73****
Absolute liver weight		100	102	**115**	**114**	100	**124**	**124**	**106**
Relative liver weight		100	106	**120**	**146****	100	**131****	**131****	**148****
Histopathology, Liver:									
Centrilobular hepatocellular swelling #, $		0/4	0/4	**3/4**	**1/4**	0/4	**3/4**	**3/4**	0
Pigment #		0/4	0/4	**3/4**	**4/4***	0/4	0/4	**3/4**	**4***
Periportal / centrilobular fibrosis #		0/4	0/4	0/4	**4/4***	0/4	0/4	0	**1/4**
Centrilobular degeneration #		0/4	0/4	**2/4**	**3/4**	0/4	0/4	**4/4***	**4/4***
Aspartate aminotransferase	4	100	52	63	**130**	100	97	131	**207***
	8	100	119	135	**208****	100	100	128	**207****
	13	100	116	113	**156****	100	103	130	**157***
Alanine aminotransferase	4	100	74	**271**	**743**	100	97	**354**	**917***
	8	100	82	**306**	**679****	100	95	**341**	**722***

Endpoints	Examination (Week)	Dose levels (ppm)							
		Male				Female			
		0	4000	12000	40000	0	4000	12000	40000
	13	100	81	**278**	**581****	100	119	**327**	**535***
Alkaline phosphatase	4	100	93	**144**	**328****	100	94	**150**	**243***
	8	100	102	**163***	**332****	100	109	**189***	**308****
	13	100	107	**174***	**294****	100	134	**229****	**349****
γ-Glutamyltranspeptidase	4	2	2	2	**6***	100	100	100	**200**
	8	2	2	2	**6**	100	100	**150**	**350**
	13	4	3	3	**10***	100	133	**167**	**267***
Triglyceride	4	100	108	140	**168**	100	91	98	**221***
	8	100	103	110	**156****	100	100	110	**151***
	13	100	126	124	**182****	100	103	111	**176***
Albumin	4	100	100	100	**85****	100	97	92	**92**
	8	100	92	94	**78****	100	100	97	**86***
	13	100	89	94	**75****	100	100	95	**78****
Globulin	13	100	110	100	**145****	100	113	113	**138**
Albumin/globulin ratios	4	100	93	93	**71****	100	88	88	**88***
	8	100	94	100	**61****	100	86	91	**68***
	13	100	89	94	**56****	100	83	83	**58****
Total cholesterol	4	100	97	97	**63****	100	93	83	97
	8	100	88	86	**49****	100	95	**80**	**80**
	13	100	82	84	**48****	100	91	**78**	**73***
Glucose	4	100	96	96	**86****	100	96	92	**85****
	8	100	106	102	**89****	100	98	102	**83****
	13	100	100	102	**79****	100	104	104	**88***

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: $p<0.05$, **: $p<0.01$. Shadow presents biologically significant change. “na” means data not available. $: Notifier considers that “centrilobular hepatocellular swelling” means “centrilobular hepatocellular hypertrophy” because “centrilobular hepatocellular hypertrophy” was recorded in the 1-year dog study.

Table 8. Summary of liver findings in the dog 1-year study

Endpoints	Examination (Week)	Dose levels (ppm)									
		Male					Female				
		0	200	800	4000	8000	0	200	800	4000	8000
Chemical intake (mg/kg/day)		0	4.3	19.2	92.0	180.7	0	4.5	20.4	92.0	225.7
Final body weight		100	104	91	96	94	100	97	96	102	84
Absolute liver weight		100	97	94	**111**	**112**	100	102	102	104	106
Relative liver weight		100	93	103	**113**	**120**	100	103	106	103	**127***
Histopathology, Liver:											
Centrilobular hepatocellular hypertrophy #		0/4	0/4	0/4	**2/4**	**3/4***	0/4	0/4	0/4	**1/4**	**4/4***
Pigment #		1/4	0/4	1	**3/4**	**4/4***	1/4	1/4	0/4	1/4	**4/4***
Pigmented macrophages #		1/4	0/4	0/4	0/4	**2/4**	1/4	0/4	0/4	1/4	**2/4**
Portal fibrosis/bile duct proliferation #		0/4	0/4	0/4	0/4	**1/4**	0/4	0/4	0/4	0/4	0/4
Centrilobular degeneration #		0/4	0/4	0/4	0/4	**1/4**	0/4	0/4	0/4	0/4	**1/4**
Aspartate aminotransferase	26	100	100	97	97	**203***	100	115	108	88	108
Alanine aminotransferase	13	100	111	116	121	**358**	100	124	131	135	**272**
	26	100	114	114	102	**269**	100	116	148	100	**184**
	52	100	133	128	118	**320**	100	121	172	114	**203**
Alkaline phosphatase	13	100	108	163	190	**283***	100	84	77	100	**227***
	26	100	98	189	226	**264***	100	105	88	104	**264****
	52	100	105	178	**273***	**359****	100	116	96	125	**336****
γ-Glutamyltranspeptidase	13	2	2	3	**3**	**4**	2	3	3	2	3
	26	3	5	3	**5**	3	2	2	4	3	4
	52	3	3	4	**4**	**4**	3	3	3	4	**5**
Albumin	26	100	97	94	97	92	100	97	92	95	**87***
	52	100	100	94	106	94	100	95	95	92	**87****
Total cholesterol	52	100	85	95	105	92	100	81	73*	79	**72***

Data presents relative values when control value is shown as 100. #: Data is scored as number of animals exhibiting findings out of total number of animals examined. *: $p<0.05$, **: $p<0.01$. Shadow presents biologically significant change. "na" means data not available.

V-4. Assessment for toxicological relevance of thyroid hypertrophy observed in general toxicity and/or carcinogenicity studies with S-2200TG

Thyroid abnormality may relate to the endocrine disrupting issue. Since the perturbation of homeostasis of the hypothalamus-pituitary-thyroid axis by an extrathyroidal mechanism appears not to be relevant to humans as discussed later, therefore, determination of MOA for possible induction of thyroid follicular-cell hypertrophy by S-2200TG in rat is important for assessment of its toxicological relevance to humans. Structured frameworks are extremely useful in promoting transparent, harmonized approaches to the risk assessment of chemicals. One area where this has

been particularly successful is in the analysis of MOAs for chemical carcinogens and other forms of toxicity in experimental animals and their relevance to humans. The International Programme on Chemical Safety (IPCS) recently published an updated version of its MOA framework in animals to address human relevance (cancer human relevance framework, or HRF)(Boobis et al., 2006). This work has now been extended to noncancer effects, with the eventual objective of harmonizing framework approaches to both cancer and noncancer endpoints (Boobis et al., 2008; Carmichael et al., 2011). Using this framework, we evaluate potential hazard of S-2200TG to humans based on MOA on thyroid.

A. Postulated mode of action for the induction of thyroid follicular-cell hypertrophy in rat

The postulated MOA for possible induction of thyroid follicular-cell hypertrophy by S-2200TG involves the perturbation of homeostasis of the hypothalamus-pituitary-thyroid axis by an extrathyroidal mechanism. Specifically, S-2200TG induces hepatic T4-UGT activity, leading to enhanced catabolism of T4 by conjugation and increased biliary excretion of the conjugated hormone. The result of this enhanced liver metabolism is a decrease in serum T4 (and sometimes T3) half-life. The pituitary gland responds to a decrease in circulating serum levels of T4 by enhancing the output and serum level of TSH. Prolonged elevation of circulating TSH levels stimulates the thyroid gland to deplete its stores of thyroid hormone and continues to induce hormone production. Thus, the thyroid follicular-cells enlarge (hypertrophy). This MOA is often associated with follicular cells proliferating at an increased rate and to increase in number (hyperplasia), and thyroid hyperplasia eventually progresses to neoplasia with chronic exposure. However, chronic exposure with S-2200TG did not induce thyroid hyperplasia or neoplasia.

B. Key events in experimental animals

The sequence of key events in the MOA for effects of S-2200TG on thyroid includes: induction of hepatic UGT activity, increase in hepatic metabolism and biliary excretion of T4, decrease in serum T4 half-life and concentration, increase in circulating TSH concentration, and thyroid follicular-cell hypertrophy.

To determine whether S-2200TG works via disruption of thyroid–pituitary status by increasing hepatic clearance of circulating thyroid hormone, several parameters were evaluated in rats in a short term study (Tables 1 and 2).

i. Enzyme induction

UGT isoform induction has been shown to be associated with thyroid hypertrophy and tumour formation in rodents for phenobarbital and related compounds (Finch et al., 2006; Hiasa, et al., 1982; McClain, et al., 1988; Whysner, et al., 1996) and involves activation of nuclear receptors, particularly the CAR (Qatanani and Moore, 2005; Qatanani, et al., 2005). Hepatic CYP2B and UGT were both induced by phenobarbital *via* CAR (Deguchi, et al., 2009; Holsapple, et al., 2006; Qatanani and Moore, 2005; Qatanani, et al., 2005; Yamamoto, et al., 2004). Increases of hepatic T4-UGT activity were observed in male rat treated with 400 ppm and higher and in female rats treated with 7000 and higher of S-2200TG for 7 days (Tables 1 and 2, Figure 1).

Additional evidence that microsomal enzyme induction is occurring at biologically relevant levels is supported by the observation that there was hepatocellular hypertrophy and proliferation of SER (Tables 1 and 2, Figure 1). These are characteristic changes of microsomal enzyme inducers,

such as phenobarbital and related compounds. Thus, there is both direct and indirect evidence for microsomal enzyme induction by S-2200TG administration with biologically important consequences.

ii. Circulating levels of thyroid hormone and TSH

Induction of hepatic T4-UGT activity results in increase in hepatic metabolism and biliary excretion of T4, and consequently decreases in serum T4 half-life and concentration. While hepatic metabolism and biliary excretion of T4 were not determined, reduced serum levels of T4 and T3 and increase of serum TSH level were observed in S-2200TG-treated rats (Tables 1 and 2, Figure 3).

iii. Thyroid morphology

Increased thyroid follicular-cell hypertrophy was observed at 7000 ppm and higher in females after 7- and 14-day treatment although the findings were not clearly increased but were observed in some treated male animals (Tables 1 and 2). Increased thyroid follicular-cell hypertrophy was also observed at longer treatments such as in the 90-day study (4000 ppm and higher), in the 1-year study (7000 ppm and higher), and the 2-year study (15000 ppm). Thus, there is direct and strong evidence to support the key event of increased thyroid follicular-cell hypertrophy by S-2200TG. Especially, time course alteration of the thyroid morphology is similar to the T4-UGT inducer, PB. In contrast to PB, however, no increased incidence of thyroid follicular-cell hyperplasia or tumours was observed in rats treated with S-2200TG even at doses of the MTD (15000 ppm) for 2 years. This is most likely related to the lower potency of S-2200TG compared to PB.

Thus, based on the key events listed, biological indicators of MOA by S-2200TG should include changes in liver metabolism, alterations in hormone levels, increases in thyroid growth, and follicular-cell hypertrophy in the thyroid.

C. Dose-response relationships

To evaluate the dose-response relationship (and time-course of the response) in rats, the data for males and females treated with S-2200TG are re-summarized in Tables 9 and 10, respectively.

i. Enzyme induction

In the MOA study (Asano, 2012), after 7-day treatment, the increased T4-UGT activity was observed in a dose related manner; statistically significant increase at 400 ppm and higher in males, and slight increase at 7000 ppm and significant increase at 15000 ppm in females,.

ii. Circulating levels of thyroid hormone and TSH

For male, decreased serum levels of T4 were observed in a dose related manner; statistically significant decrease was observed at 15000 ppm after 7- and 14-day treatment. No apparent alteration was observed in T3 and TSH after 7-day treatment. However, after 14-day treatment, 15000 ppm of S-2200TG revealed statistically significant increase of TSH accompanied with

significant decrease of T4.

For females, decreased serum T4 and increased serum TSH were observed in a dose related manner; statistically significant change was not observed on T4 but observed on TSH at 15000 ppm. However, after 14-day treatment, 15000 ppm of S-2200TG significantly decreased T4 and significantly increased TSH.

iii. Thyroid morphology

For males, a small number of animals (3-5 out of 10) revealed thyroid follicular-cell hypertrophy after 7- or 14-day treatment at 15000 ppm but the incidence was not statistically significant from control (1-2 out of 10). However, after 90-day treatment, the finding was increased at 4000 ppm (6 out of 12, without statistical significance), 10000 ppm (9 out of 12, with statistical significance), and 20000 ppm (7 out of 12, without statistical significance). After longer treatment, significant increase was observed at 7000 and 15000 ppm after 1-year treatment and at 15000 ppm after 2-year treatment. Overall, the Lowest Observable Effect Level (LOEL) on thyroid follicular-cell hypertrophy was considered to be 4000 ppm in males.

For females, thyroid follicular-cell hypertrophy was significantly increased at 7000 ppm and higher after 7-day treatment. Small number of animals (4-6 out of 11-12) revealed thyroid follicular-cell hypertrophy at 7000 ppm and higher but the incidence was not statistically significant from control incidence (2 out of 12) after 90-day treatment. Regarding thyroid follicular-cell hypertrophy observed in small number of animals (4 out of 11) at 4000 ppm, it is difficult to consider it as biologically relevant because liver hypertrophy (increased liver weight and hepatocellular hypertrophy) at 4000 ppm was much less potent than those at 10000 ppm. After longer treatment, a significant increase was observed at 7000 and 15000 ppm after 1-year treatment, and no significant increase was observed after 2-year treatment (only 4 of 50 animals had the finding at 15000 ppm). Overall, the LOEL on thyroid follicular-cell hypertrophy was considered to be 7000 ppm in females.

The effects on liver enzymes/weight and pituitary–thyroid hormone concentrations would be anticipated to occur at doses at least as low as those that produce thyroid weight changes and increases in thyroid follicular-cell hypertrophy incidence, given that this thyroid disruption MOA is a threshold phenomenon (Dellarco et al., 2006). Although we should consider phase of treatment as discussed in *"Temporal association" Section*, the various parameters described above that are related to the key events in the development of thyroid follicular-cell hypertrophy were generally observed at or below the dose levels producing hypertrophy (Tables 9 and 10). In addition, as discussed later, the alterations of the biochemical key events preceded to the morphological alterations, as evidenced by early phase of treatment in males.

Taken together, there are strong parallels in the dose response for the key events and thyroid follicular-cell hypertrophy. Again, it is noteworthy that the degree of change observed in each of the parameters was relatively mild, consistent with no increase of hypertrophy incidences. However, the increased incidence of thyroid follicular-cell hyperplasia and neoplasia was not observed in rats treated with S-2200TG at 15000 ppm for 2 years.

Table 9. Dose-response relationship of alteration on thyroid and liver of male rats treated with S-2200TG

Treatment	Dose levels (ppm)									
period	0	400	800	2000	4000	7000	10000	15000	20000	PB
Hepatocellular hypertrophy (number of animals with finding /number of animals examined)										
7 days	0/10	0/10	na	1/10	na	8/10**	na	9/10**	na	10/10**
14 days	2/10	na	na	na	na	na	na	10/10**	na	10/10**
90 days	0/12	na	0/12	na	12/12	na	12/12	na	12/12	na
1 year	0/19	0/19	na	0/19	na	15/20**	na	20/20**	na	na
2 years	7/50	11/50	na	13/50	na	30/50**	na	37/50**	na	na
Relative liver weight (g/100gBW)										
7 days	3.65±0.22	3.61±0.177	na	3.77±0.214	na	4.03±0.215**	na	4.53±0.342**	na	4.51±0.278**
	100	99	na	103	na	110	na	124	na	124
14 days	3.47±0.223	na	na	na	na	na	na	4.52±0.232**	na	4.68±0.201**
	100	na	na	na	na	na	na	130	na	135
90 days	2.262± 0.0711	na	2.368± 0.1446	na	2.607± 0.0993**	na	2.885± 0.1368**	na	3.541± 0.2167**	na
	100	na	105	na	115	na	128	na	157	na
1 year	2.014± 0.1662	2.157± 0.1906	na	2.218± 0.2171*	na	2.350± 0.159**	na	2.816± 0.3355**	na	na
	100	107	na	110	na	117	na	140	na	na
2 years	2.051± 0.1704	2.029± 0.187	na	2.042± 0.2167	na	2.215± 0.2068	na	2.334± 0.2452*	na	na
	100	99	na	100	na	108	na	114	na	na
Hepatic T4 UGT induction (pmol/min/mg S9 protein)										
7 days	0.41±0.054	0.51±0.024*	na	0.54±0.075	na	0.62±0.051*	na	0.61±0.046*	na	0.79±0.122*
	100	124	na	132	na	151	na	149	na	193
Serum T3 levels (ng/mL)										
7 days	0.5±0.07	0.5±0.1	na	0.5±0.08	na	0.4±0.08	na	0.5±0.1	na	0.5±0.11
	100	100	na	100	na	80	na	100	na	100
14 days	0.5±0.11	na	na	na	na	na	na	0.4±0.1	na	0.5±0.08
	100	na	na	na	na	na	na	80	na	100
Serum T4 levels (μg/dL)										
7 days	5.04±0.722	5.35±0.848	na	5.36±0.71	na	4.56±0.897	na	4.09±0.869*	na	4.45±1.305
	100	106	na	106	na	90	na	81	na	88
14 days	5.7±0.744	na	na	na	na	na	na	4.08±0.978**	na	4.03±0.872**
	100	na	na	na	na	na	na	72	na	71
Serum TSH levels (ng/mL)										
7 days	8.6±2.76	7.3±2.33	na	7.5±2.5	na	6.7±2.12	na	7.5±2.96	na	10.6±3.42
	100	85	na	87	na	78	na	87	na	123
14 days	8.0±3.75	na	na	na	na	na	na	11.7±3.6*	na	12.7±3.9*
	100	na	na	na	na	na	na	146	na	159
Relative thyroid weight (mg/100g BW)										
7 days	5.2±0.74	5.6±0.98	na	5.8±1.58	na	5.4±0.88	na	6.2±0.92	na	6.2±1.43
	100	108	na	112	na	104	na	119	na	119
14 days	6.4±0.83	na	na	na	na	na	na	7.5±1.23*	na	8.1±0.9**
	100	na	na	na	na	na	na	117	na	127
90 days	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
1 year	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
2 years	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
Throid folicullar-cell hypertrophy; diffuse (number of animals with finding /number of animals examined)										
7 days	1/10	1/10	na	2/10	na	2/10	na	3/10	na	5/10
14 days	2/10	na	na	na	na	na	na	5/10	na	6/10
90 days	2/12	na	2/12	na	6/12	na	9/12*	na	7/12	na
1 year	1/19	0/19	na	1/19	na	9/20**	na	18/20**	na	na
2 years	1/50	2/50	na	0/50	na	0/50	na	11/50**	na	na
Thyroid follicular-cell adenoma (number of animals with finding /number of animals examined)										
2 years	7/50	4/50	na	7/50	na	7/50	na	7/50	na	na
Thyroid follicular-cell carcinoma (number of animals with finding /number of animals examined)										
2 years	0/50	0/50	na	2/50	na	1/50	na	0/50	na	na

Data presents mean ± SD and relative values when control value is shown as 100. *: p<0.05, **: p<0.01. Shadow presents biologically significant change. "na" means data is not available.

Table 10. Dose-response relationship of alteration on thyroid and liver of female rats treated with S-2200TG

Treatment	Dose levels (ppm)									
period	0	400	800	2000	4000	7000	10000	15000	20000	PB
Hepatocellular hypertrophy (number of animals with finding /number of animals examined)										
7 days	1/10	1/10	na	5/10	na	6/10*	na	8/10**	na	10/10**
14 days	0/10	na	na	na	na	na	na	5/10*	na	10/10**
90 days	0/12	na	0/12	na	4/11*	na	9/12**	na	12/12**	na
1 year	0/20	1/20	na	1/19	na	17/19**	na	15/20**	na	na
2 years	16/50	22/50	na	32/50**	na	43/50**	na	42/50**	na	na
Relative liver weight (g/100gBW)										
7 days	3.44±0.125	3.41±0.241	na	3.63±0.12*	na	3.79±0.095**	na	4.17±0.118**	na	4.07±0.232**
	100	99	na	106	na	110	na	121	na	118
14 days	3.24±0.197	na	na	na	na	na	na	4.14±0.187**	na	4.12±0.304**
	100	na	na	na	na	na	na	128	na	127
90 days	2.550± 0.2289	na	2.510± 0.1422	na	2.760± 0.1770	na	3.047± 0.2271**	na	3.814± 0.2856**	na
	100	na	98	na	108	na	119	na	150	na
1 year	2.328± 0.2131	2.335± 0.2476	na	2.439± 0.2162	na	2.826± 0.4013**	na	3.150± 0.3017**	na	na
	100	100	na	105	na	121	na	135	na	na
2 years	2.153± 0.3228	2.038± 0.2232	na	2.472± 0.2223*	na	2.446± 0.209*	na	2.753± 0.1907**	na	na
	100	95	na	115	na	114	na	128	na	na
Hepatic T4 UGT induction (pmol/min/mg S9 protein)										
7 days	0.46±0.069	0.43±0.033	na	0.46±0.071	na	0.53±0.057	na	0.62±0.074**	na	0.56±0.06*
	100	93	na	100	na	115	na	135	na	122
Serum T3 levels (ng/mL)										
7 days	0.6±0.19	0.6±0.12	na	0.6±0.21	na	0.6±0.15	na	0.6±0.08	na	0.6±0.14
	100	100	na	100	na	100	na	100	na	100
14 days	0.7±0.15	na	na	na	na	na	na	0.5±0.88**	na	0.5±0.11**
	100	na	na	na	na	na	na	71	na	71
Serum T4 levels (µg/dL)										
7 days	4.7±1.412	4.7±1.124	na	4.52±1.331	na	4.34±1.24	na	3.95±1.311	na	3.17±0.641**
	100	100	na	96	na	92	na	84	na	67
14 days	4.68±1.152	na	na	na	na	na	na	3.5±0.718*	na	2.52±0.572**
	100	na	na	na	na	na	na	75	na	54
Serum TSH levels (ng/mL)										
7 days	6.1±1.71	6.3±2.13	na	6.7±2.14	na	9.1±4.99	na	15.1±6.22**	na	10.2±3.8**
	100	103	na	110	na	149	na	248	na	167
14 days	6.1±1.97	na	na	na	na	na	na	12.3±6.55*	na	10.6±4.48*
	100	na	na	na	na	na	na	202	na	174
Relative thyroid weight (mg/100g BW)										
7 days	6.5±1.38	6.9±1.09	na	6.7±1.17	na	8.6±1.12**	na	8.5±1.38**	na	7.9±1.04*
	100	106	na	103	na	132	na	131	na	122
14 days	6.7±1.01	na	na	na	na	na	na	8.9±1.05**	na	8.1±1**
	100	na	na	na	na	na	na	133	na	121
90 days	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
1 year	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
2 years	na	na	na	na	na	na	na	na	na	na
	na	na	na	na	na	na	na	na	na	na
Throid folicullar-cell hypertrophy; diffuse (number of animals with finding /number of animals examined)										
7 days	0/10	1/10	na	1/10	na	4/10*	na	6/10**	na	3/10
14 days	0/10	na	na	na	na	na	na	5/10*	na	3/10
90 days	2/12	na	1/12	na	4/11	na	5/12	na	6/12	na
1 year	0/20	0/20	na	0/19	na	9/19**	na	15/20**	na	na
2 years	0/50	1/50	na	2/50	na	0/50	na	4/50	na	na
Thyroid follicular-cell adenoma (number of animals with finding /number of animals examined)										
2 years	2/50	2/50	na	2/50	na	5/50	na	1/50	na	na
Thyroid follicular-cell carcinoma (number of animals with finding /number of animals examined)										
2 years	0/50	0/50	na	0/50	na	1/50	na	0/50	na	na

Data presents mean ± SD and relative values when control value is shown as 100. *: $p<0.05$, **: $p<0.01$. Shadow presents biologically significant change. "na" means data is not available.

D. Temporal association

It is critical in the evaluation of the MOA that early key events occur before the appearance of thyroid follicular-cell hypertrophy, and this is clearly the case with S-2200TG. Multiple exposure time data at 7, 14, and 90 days, and 1 and 2 years are available in which Wistar rats were administered diets containing 15000 ppm (Tables 9 and 10). Liver effects, including weight and hepatocellular hypertrophy, were increased at all observation times from the earliest time of assessment on day 7. Hepatic T4-UGT activity was also increased at the earliest observation time

examined (day 7). Biliary excretion of conjugated T4 was not measured in this experiment; however, serum T4 was reduced at an early phase of treatment, such as days 7 and/or 14. Although the increase was not statistically significant in males but was in females, the increases in circulating TSH were observed in both sexes at days 7 and/or 14. Slight but continuous increase of circulating TSH is considered enough to alter thyroid morphology such as follicular-cell hypertrophy and hyperplasia (Hood et al., 1999). Increases in thyroid weight were also observed at 7 and/or 14 days after treatment began. Histopathologically, there was a time-related increase in follicular-cell hypertrophy beginning at 7 or 14 days. Thus, there is a logical temporal response for the key events of formation for thyroid follicular-cell hypertrophy in which all key events precede the hypertrophy induction as clearly evident especially in males.

Generally, the follicular-cell hyperplasia and tumour occurred only after chronic administration of UDP inducers (Finch et al., 2006; Hurley et al., 1998; IARC, 2001; Whysner,1996). But in the case of S-2200TG, hyperplastic and neoplastic changes were not observed even after 2-year treatment.

E. Strength, consistency, and specificity of association of the response with key events

Strength, consistency, and specificity of the association can be established from the studies described earlier. The quantifiable precursor events, fundamental to the proposed MOA, are relatively consistent with the emergence of thyroid follicular-cell hypertrophy. Observation of liver weight increase and induction of hepatic T4-UGT in rats receiving S-2200TG in the diet would be consistent with perturbation of homeostasis of the pituitary–thyroid axis by an extrathyroidal mechanism. An increase in hepatic T4-UGT activity is a step occurring before the other key biochemical changes and before thyroid follicular-cell hypertrophy. S-2200TG treatment clearly results in a decrease in circulating T4 and an increase in TSH following enhanced liver metabolism of T4. Furthermore, the development of thyroid hypertrophy was shown to be increased under the same conditions of dose and time as the appearance of changes in thyroid hormone levels.

F. Biological plausibility and coherence

As mentioned earlier in this document, there is an excellent review of thyroid biology (Dellarco, et al., 2006; IARC, 2001). The published data are consistent with increased TSH concentrations alone causing thyroid follicular-cells of rats to enter a state of pre-hypertrophy. Therefore, the suggestion that high dose of S-2200TG causes thyroid follicular-cell hypertrophy in rats by initially inducing hepatic T4-UGT is coherent with the known physiology of the hypothalamus–pituitary–thyroid dynamic control system, at least to the stage of hypertrophy. The S-2200TG-induced key events summarized above are similar to those seen with PB (Dellarco, et al., 2006; Finch, et al., 2006), and we can therefore conclude that S-2200TG has a similar MOA to that of CAR activators such as PB for rat thyroid hypertrophy formation.

G. Other modes of action

As described in the earlier discussion, it can be concluded that S-2200TG has no genotoxic potential, suggesting that the MOA for thyroid follicular-cell hypertrophy is non-genotoxic.

Additional effects on the hypothalamic–pituitary–thyroid axis or disruption of other pathways of thyroid hormone metabolism (ex., blockade of T4 synthesis, receptor blockade etc) are other possibilities of altering thyroid homeostasis. These variations would not differ in any fundamental way from the one that has been proposed for S-2200TG; all would lead to prolonged TSH stimulation with continuous exposure. Furthermore, we should recognize that S-2200TG did not

induce direct cytotoxicity on the thyroid as evidenced by histopathology.

H. Uncertainties, inconsistencies, and data gaps

For the most part, the data are consistent with the proposed key events at several time points and at several doses, as noted in Tables 7 and 8. However, there are some apparent gaps and inconsistencies.

The increased thyroid weight is a key event in the MOA for thyroid hypertrophy induced by enhancement of thyroid hormone metabolizing enzyme (Dellarco, et al., 2006). However, the thyroid weight was not fully examined in all studies with S-2200TG; it was not examined in the rat 90-day and chronic/carcinogenicity studies. Data of thyroid weight is sometimes unreliable because of its small size. Thyroid histopathology tends to be a more reliable indicator than thyroid weight (DeVito et al., 1999; Hood, et al., 1999; Yamada et al., 2004). Therefore, we do not believe that this is an essential data gap because direct evidence of histopathological alterations of thyroid, such as hypertrophy, suggesting increased thyroid weight, was observed.

Although data for T4 biliary elimination is not collected, we believe that this is not essential because the direct evidence for reduced serum T4 and of increased hepatic T4-UGT by S-2200TG administration was obtained.

Lastly, compared to the typical rodent thyroid carcinogens (thyroid follicular-cell tumour inducer such as CAR activators, PB and pyrethrin) (Finch et al., 2006; IARC, 2001), a thyroid tumour response was not elicited by S-2200TG although S-2200TG has CAR activation evidenced by CYP2B induction and proliferation of SER in hepatocytes (Asano, 2012). Although CAR activators often induce liver tumour in rodents (IARC, 2001; Osimitz and Lake, 2009; Yamada, et al., 2009), S-2200TG did not induce liver tumours in rat or mouse. Therefore, these findings suggest that CAR activation by S-2200TG may attenuate at later phases of treatment or may lack unknown key event(s) which are necessary for cell transformation. Most likely, the low potency of S-2200TG compared to PB and that reported for other CAR activators is the basis for its mild effect on liver thyroid and the lack of tumourigenicity in either organ. While details are unknown, the lack of tumourigenicity decreases toxicological concern of the thyroid and liver hypertrophy caused by S-2200TG.

I. Assessment of postulated mode of action

As described above, the key events in the MOA for S-2200TG have been well documented, with a strong dose and temporal consistency. In addition, this is a well known MOA, and the various parameters essential for documenting this MOA have been presented for S-2200TG. Thus, we consider that the level of confidence in the postulated MOA is high.

J. Human applicability of the proposed mode of action

Human applicability of the proposed MOA for thyroid follicular-cell hypertrophy by S-2200TG is also evaluated by the IPCS Human Relevance Framework (Boobis, et al., 2008; Carmichael, et al., 2011).

1. Is the weight of evidence sufficient to establish a mode of action in animals?

As described in detail earlier, there is clear evidence that S-2200TG alters thyroid homeostasis by UDP induction causing reduced serum T4 (and maybe T3) levels and consequently elevating

serum TSH.

2. *Can human relevance of the mode of action be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?*

The current understanding of the regulation of thyroid hormone homeostasis in humans and of the role of increased TSH levels (as a result of altered thyroid homeostasis) as a risk factor for thyroid abnormality including cancer was considered in order to assess the human relevance of the key events in animal MOA by S-2200TG. Although there are substantial quantitative dynamic differences (discussed later), the fundamental mechanisms involved in the function and regulation of the hypothalamic–pituitary–thyroid axis in rats are qualitatively similar to those in humans (Bianco et al., 2002). UGT activation by CAR activators is also operated in human (Sugatani, et al., 2001). Therefore, an agent that decreases T4 levels in rats could likewise reduce T4 in humans; this, in turn, could potentially lead to an increase in TSH levels. There are data showing that rodents and humans respond in a similar fashion to perturbations of pituitary–thyroid function. For example, it is well known that iodine deficiency, which readily leads to decreased thyroid hormone levels, stimulates thyroid cell proliferation in humans, leading to goiter. If left untreated, iodine deficiency may lead to tumour formation, albeit rarely (Thomas and Williams, 1999). Although there is no evidence of increased susceptibility to thyroid cancer, a number of pharmaceuticals (e.g., propylthiouracil, lithium, amiodarone, iopanoic acid) that disrupt thyroid homeostasis by acting directly on the thyroid gland (for example, by inhibiting hormone synthesis or release or by blocking the conversion of T4 to T3) are known to lead to hypothyroidism and increases in TSH in humans (Ron et al., 1987).

In contrast to rats, no increases in TSH levels have been found in humans following exposure to agents that induce hepatic microsomal enzymes and reduce circulating T4 levels (Meek, et al., 2003). For example, the pharmaceutical compounds phenytoin, rifampin, and carbamazepine induce hepatic microsomal enzymes, including UGT, and reduce circulating T4 levels, but TSH levels are unchanged (Curran and DeGroot, 1991); agents that produce thyroid tumours in rats by increasing glucuronidation and biliary excretion of T4 at high experimental doses (e.g., omeprazole, lansoprazole, and pantoprazole) produce no changes in thyroid hormones at clinical doses in humans (Masubuchi et al., 1997). Thus, there appears to be a substantial difference in the dose-response relationship for altered homeostasis of the pituitary–thyroid axis in rats compared to humans. As discussed next, this observation is due to quantitative dynamic differences between rats and humans in the basic physiological processes underlying pituitary–thyroid function.

3. Can human relevance of the mode of action be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

The primary effect of S-2200TG is on hepatic metabolizing enzymes, and the increase in metabolic activity indirectly increases the systemic clearance of T4 (and maybe T3), leading to the hypothyroid state and the compensatory increase in TSH found in rats. Although there are no chemical-specific data on the potential for S-2200TG to disrupt thyroid hormone homeostasis in humans, a number of other microsomal enzyme inducers have been extensively studied, such as phenobarbital (Meek, et al., 2003). UGT activation by CAR activators is operated in human (Sugatani, et al., 2001). However, as discussed earlier, agents that produce hypothyroidism by altering hepatic clearance of T4 do not appear to result in elevated TSH levels in humans. Presumably, TSH is not increased because a critical reduction of T4 is not reached. There are

several important physiological and biochemical differences between rats and humans related to thyroid function. Rats have a smaller reserve capacity of thyroid hormones when compared with humans. The rat has a much shorter thyroid hormone half-life than humans. The half life of T4 is about 12 h in rats, compared to 5–9 days in humans (Dohler et al., 1979). The shorter half-life in rats is likely related to the absence of a high-affinity binding globulin for T4 that is present in humans (Hill, et al., 1989). In rats, the increased clearance contributes to the need for a higher rate of production of T4 (per unit of body weight) to maintain normal levels of T4. In contrast, in humans, the binding of thyroid hormone to this globulin accounts for a slower metabolic degradation and clearance that in turn results in the thyroid gland being less active than in rats. The constitutive TSH levels are approximately 25 times higher in rats than in humans, reflecting the higher activity of the pituitary–thyroid axis in rats (Dohler, et al., 1979; McClain, 1992). Therefore, humans are quantitatively less sensitive than rats to agents that reduce T4 and lead to elevated TSH. There is no increased risk of thyroid tumour development if TSH is not elevated.

Another difference of rats compared to humans is the histological appearance of the thyroid. This histological difference is related to the higher rate of production of T4 to maintain a consistent serum concentration, thus making the rat thyroid more "functionally active" than primates including humans (McClain, 1995). More of the follicular epithelium in the rat is stimulated to synthesize thyroglobulin, and therefore more of the follicular-cells are tall cuboidal and appear to be active in synthesis. In contrast, more of the follicular-cells in humans tend to be short cuboidal or almost squamous in appearance, suggesting they are quiescent. Because rat follicular-cells are already generally active, under stimulation from TSH, they will respond with hyperplasia more readily than human follicular-cells. Because of the greater storage capability of the human thyroid and the greater numbers of cells in a quiescent state, human thyroid follicular-cells will be roused from their quiescent state to synthesize and secrete additional thyroid hormone without the need for a hyperplastic response to reestablish homeostasis. Therefore, the primary response in the human thyroid gland would be thyroglobulin reabsorption and cellular hypertrophy rather than hyperplasia. In short, there is much greater buffering capacity in the biochemistry of the human than the rat thyroid.

Even though certain agents can cause a reduction in thyroid hormone levels in humans, there is no clear evidence that these agents increase susceptibility to thyroid cancer (Ron, et al., 1987). For example, epidemiologic studies with phenobarbital do not show any increased risk of thyroid cancer (Friedman et al., 2009; Olsen, et al., 1989; Olsen et al., 1993). Studies of individuals with conditions that would lead to elevated TSH (patients with Graves' disease or goiter) indicate the occurrence of thyroid cancer is rare in these circumstances (Gabriele et al., 2003; Mazzaferri, 2000). A study of environmental and heritable causes of cancer among 9.6 million individuals, using the Nationwide Swedish Family Cancer Database, found that the environment did not appear to play a principal causative role in thyroid cancer (Czene et al., 2002). The only known human thyroid carcinogen is radiation, a mutagenic exposure. As summarized in Table 11, there is sufficient evidence in the general literature on the biochemical and physiological differences in thyroid function to indicate differences in toxicity susceptibility including tumour induction between rats and humans. In contrast to humans, rats are very susceptible to thyroid abnormality secondary to hypothyroidism. In particular, modest changes in thyroid hormone homeostasis will induce hypertrophy and promote tumour formation in rats. Thus, thyroid follicular-cell hypertrophy observed in rats treated with 7000 ppm of S-2200TG involving increased hepatic clearance of hormone and altered homeostasis of the pituitary–thyroid axis in rodents are considered not relevant to humans, based on quantitative dynamic differences. Even if S-2200TG induce thyroid follicular-cell hypertrophy in human, no tumour is developed in human.

Table 11. A comparison of key events by S-2200TG in rats and human

Key event	Evidence in rats	Evidence in humans
Increase hepatic clearance of T4.	In short-term and chronic rat studies, the liver is found to be the most sensitive target, and evidence of increased T4 hepatic clearance is provided by studies on T4-hepatic UGT activity, and liver weights and hepatocellular hypertrophy.	No data available for S-2200TG, but microsomal enzyme induction is plausible.
Decreased serum T4.	Direct experimental evidence.	No data available for S-2200TG, but plausible given that other microsomal enzyme inducers have been shown to reduce T4 in humans.
Increased TSH levels.	Direct experimental evidence.	No data available for S-2200TG, but other microsomal enzyme inducers have not been shown to increase TSH levels even when T4 is decreased.
Increased TSH increases thyroid cell hypertrophy formation.	Direct experimental evidence.	Induction of thyroid follicular-cell hypertrophy secondary to hypothyroidism is remote in humans, given the quantitative differences in thyroid function/homeostasis.
Increased TSH increases thyroid cell proliferation (hyperplasia) and tumour formation.	Direct experimental evidence showing no thyroid hyperplasia and tumour development.	Induction of thyroid follicular-cell tumours secondary to hypothyroidism is remote in humans, given the quantitative differences in thyroid function/homeostasis. Occurrence of thyroid cancer is rare even in severely hypothyroid individuals.

K. Conclusion: statement of confidence, analysis, and implications

The data available for S-2200TG are considerable, and, despite some data gaps such as the lack of thyroid weight data and direct data regarding T4 biliary elimination, it is clear that the MOA for S-2200TG-induced rat thyroid follicular-cell hypertrophy is secondary to enhanced metabolism of T4 (and maybe T3) leading to hormone imbalance. Although the possibility that S-2200TG may potentially result in hypothyroidism in humans can not be ruled out, there is sufficient quantitative evidence on the basic physiological processes in the general literature to conclude that thyroid abnormality including tumours induced by a process involving increased hepatic clearance of thyroid hormone and altered homeostasis of the hypothalamus–pituitary–thyroid axis in rodents is not likely to lead to an increase in susceptibility to thyroid abnormality development (including tumour) in humans. Based on the above evidence, it is reasonable to conclude that S-2200TG will not have any hazard on thyroid in humans.

VI. Conclusions

In this assessment, we firstly conclude that S-2200TG is a hepatic enzyme inducer via at least CAR activation in rat, similar to PB, evidenced by CYP2B and UGT induction and SER proliferation. Therefore, the liver hypertrophy (i.e., increased liver weight and/or hepatocellular

hypertrophy) caused by S-2200TG is judged to be an adaptive response by enzyme induction at least via CAR and not adverse. This activity also appears to be plausible in mouse and dog. Furthermore, this activity would theoretically operate in humans, as demonstrated by CYP2B inducers. S-2200TG at much higher dose levels induced adverse effects on the liver as some additional functional changes or additional pathological findings to the hypertrophy were observed. However, the adverse effects occurred in a dose related manner and there was a threshold at relatively high exposure levels; and most importantly, S-2200TG did not induce liver tumours in rat and mouse which further reduces concern in human risk assessment.

Secondly, we obtained data indicating that S-2200TG increased T4-UGT and indirectly perturbed the hypothalamus-pituitary-thyroid hormone axis, and then increased thyroid follicular-cell hypertrophy in rats, which is also similar to PB, a CAR activator. The postulated MOA for possible induction of thyroid follicular-cell hypertrophy in rats was tested against the modified Bradford Hill criteria, and was found to satisfy the conditions of dose and temporal concordance, biological plausibility, coherence, strength, consistency, and specificity that fit with a well-established MOA for thyroid follicular-cell hypertrophy. Although the postulated MOA could theoretically qualitatively operate in humans, marked quantitative differences in the inherent susceptibility for thyroid abnormality, especially tumour induction, to thyroid hormone imbalance in rats.

Therefore, even through liver and/or thyroid hypertrophy were induced by S-2200TG treatment in experimental animals, the findings from MOA analysis allow for the conclusion that S-2200TG does not pose a hazard to humans.

VII. References

Asano, K. (2012). Short-term study for mode of action analysis for rat liver and thyroid findings by S-2200TG-Dose response, time-course, and reversibility-. Sumitomo Chemical Company Ltd., Study No. S1560, Technical report ROT-0067, Unpublished data.

Bayer, I., Mitmaker, B., Gordon, P. H. and Wang, E. (1992). Modulation of nuclear statin expression in rat thyroid follicle cell following administration of thyroid stimulating hormone. *J. Cell. Physiol.* **150**, 276-282.

Bianco, A. C., Salvatore, D., Gereben, B., Berry, M. J. and Larsen, P. R. (2002). Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr. Rev.* **23**, 38-89.

Boobis, A. R., Cohen, S. M., Dellarco, V., McGregor, D., Meek, M. E., Vickers, C., Willcocks, D. and Farland, W. (2006). IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36**, 781-792.

Boobis, A. R., Doe, J. E., Heinrich-Hirsch, B., Meek, M. E., Munn, S., Ruchirawat, M., Schlatter, J., Seed, J. and Vickers, C. (2008). IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Crit. Rev. Toxicol.* **38**, 87-96.

Capen, C. C. (1997). Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. *Toxicol. Pathol.* **25**, 39-48.

Carmichael, N., Bausen, M., Boobis, A. R., Cohen, S. M., Embry, M., Fruijtier-Polloth, C., Greim, H., Lewis, R., Meek, M. E., Mellor, H., Vickers, C. and Doe, J. (2011). Using mode of action information to improve regulatory decision-making: An ECETOC/ILSI RF/HESI workshop overview. *Crit. Rev. Toxicol.* **41**, 175-186.

Cohen, S. M. and Arnold, L. L. (2011). Chemical Carcinogenesis. *Toxicol. Sci.* **120**, S76-S92.

Curran, P. G. and DeGroot, L. J. (1991). The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. *Endocr. Rev.* **12**, 135-150.

Czene, K., Lichtenstein, P. and Hemminki, K. (2002). Environmental and heritable causes of cancer among 9.6 million individuals in the swedish family-cancer database. *Int. J. Cancer* **99**, 260-266.

Deguchi, Y., Yamada, T., Hirose, Y., Nagahori, H., Kushida, M., Sumida, K., Sukata, T., Tomigahara, Y., Nishioka, K., Uwagawa, S., Kawamura, S. and Okuno, Y. (2009). Mode of action analysis for the synthetic pyrethroid metofluthrin-induced rat liver tumours: Evidence for hepatic CYP2B induction and hepatocyte proliferation. *Toxicol. Sci.* **108**, 69-80.

Dellarco, V. L., McGregor, D., Berry, S. C., Cohen, S. M. and Boobis, A. R. (2006). Thiazopyr and thyroid disruption: Case study within the context of the 2006 IPCS human relevance framework for analysis of a cancer mode of action. *Crit. Rev. Toxicol.* **36**, 793-801.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A. O., Christensen, R., Colborn, T., Cooke, P., Crissman, J., Crofton, K., Doerge, D., Gray, E., Hauser, P., Hurley, P., Kohn, M., Lazar, J., McMaster, S., McClain, M., McConnell, E., Meier, C., Miller, R., Tietge, J. and Tyl, R. (1999). Screening methods for thyroid hormone disruptors. *Environ. Health Perspect.* **107**, 407-415.

Dohler, K. D., Wong, C. C. and von zur Muhlen, A. (1979). The rat as model for the study of drug effects on thyroid function: Consideration of methodological problems. *Pharmacol. Ther. [B]*. **5**, 305-318.

Finch, J. M., Osimitz, T. G., Gabriel, K. L., Martin, T., Henderson, W. J., Capen, C. C., Butler, W. H. and Lake, B. G. (2006). A mode of action for induction of thyroid gland tumours by pyrethrins in the rat. *Toxicol. Appl. Pharmacol.* **214**, 253-262.

Friedman, G. D., Jiang, S.-F., Udaltsova, N., Quesenberry, C. P., Chan, J. and Habel, L. A. (2009). Epidemiologic evaluation of pharmaceuticals with limited evidence of carcinogenicity. *Int. J. Cancer* **125**, 2173-2178.

Gabriele, R., Letizia, C., Borghese, M., De Toma, G., Celi, M., Izzo, L. and Cavallaro, A. (2003). Thyroid cancer in patients with hyperthyroidism. *Horm. Res.* **60**, 79-83.

Hard, G. C. (1998). Recent developments in the investigation of thyroid regulation and thyroid carcinogenesis. *Environ. Health Perspect.* **106**, 427-436.

Hiasa, Y., Kitahori, Y., Ohshima, M., Fujita, T., Yuasa, T., Konishi, N. and Miyashiro, A. (1982). Promoting effects of phenobarbital and barbital on development of thyroid tumours in rats treated with n-bis(2-hydroxypropyl)nitrosamine. *Carcinogenesis* **3**, 1187-1190.

Hill, R. N., Erdreich, L. S., Paynter, O. E., Roberts, P. A., Rosenthal, S. L. and Wilkinson, C. F. (1989). Thyroid follicular cell carcinogenesis. *Fundam. Appl. Toxicol.* **12**, 629-697.

Holsapple, M. P., Pitot, H. C., Cohen, S. M., Boobis, A. R., Klaunig, J. E., Pastoor, T., Dellarco, V. L. and Dragan, Y. P. (2006). Mode of action in relevance of rodent liver tumours to human cancer risk. *Toxicol. Sci.* **89**, 51-56.

Hood, A., Liu, Y. P., Gattone, V. H., 2nd and Klaassen, C. D. (1999). Sensitivity of thyroid gland growth to thyroid stimulating hormone (TSH) in rats treated with antithyroid drugs. *Toxicol. Sci.* **49**, 263-271.

Hurley, P. M., Hill, R. N. and Whiting, R. J. (1998). Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumours in rodents. *Environ. Health Perspect.* **106**, 437-445.

IARC (2001). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. Some Thyrotropic Agents. Lyon, France.

JMPR (2006). Joint FAO/WHO Meeting on Persicide Residues, Summary Report. 5. Guidance on the interpretation of hepatocellular hypertrophy.

Jones, H. B., Orton, T. C. and Lake, B. G. (2009). Effect of chronic phenobarbitone administration on liver tumour formation in the C57BL/10J mouse. *Food Chem. Toxicol.* **47**, 1333-40.

Klaunig, J. E., Babich, M. A., Baetcke, K. P., Cook, J. C., Corton, J. C., David, R. M., DeLuca, J. G., Lai, D. Y., McKee, R. H., Peters, J. M., Roberts, R. A. and Fenner-Crisp, P. A. (2003). PPARalpha agonist-induced rodent tumors: modes of action and human relevance. *Crit. Rev. Toxicol.* **33**, 655-780.

Masubuchi, N., Hakusui, H. and Okazaki, O. (1997). Effects of proton pump inhibitors on thyroid hormone metabolism in rats : A comparison of UDP-glucuronyltransferase induction. *Biochem. Pharmacol.* **54**, 1225-1231.

Mazzaferri, E. L. (2000). Thyroid cancer and Graves' disease: The controversy ten years later. *Endocr Pract* **6**, 221-225.

McClain, R. M. (1992). Thyroid gland neoplasia: Non-genotoxic mechanisms. *Toxicol. Lett.* **64-65** Spec No, 397-408.

McClain, R. M. (1995). Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. *Mutat. Res.* **333**, 131-142.

McClain, R. M., Posch, R. C., Bosakowski, T. and Armstrong, J. M. (1988). Studies on the mode of action for thyroid gland tumour promotion in rats by phenobarbital. *Toxicol. Appl. Pharmacol.* **94**, 254-265.

Meek, M. E., Bucher, J. R., Cohen, S. M., Dellarco, V., Hill, R. N., Lehman-McKeeman, L. D., Longfellow, D. G., Pastoor, T., Seed, J. and Patton, D. E. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol.* **33**, 591-653.

Olsen, J. H., Boice, J. D., Jr., Jensen, J. P. and Fraumeni, J. F., Jr. (1989). Cancer among epileptic patients exposed to anticonvulsant drugs. *J. Natl. Cancer Inst.* **81**, 803-808.

Olsen, J. H., Wallin, H., Boice, J. D., Jr., Rask, K., Schulgen, G. and Fraumeni, J. F., Jr. (1993). Phenobarbital, drug metabolism, and human cancer. *Cancer Epidemiol. Biomarkers Prev.* **2**, 449-452.

Osimitz, T. G. and Lake, B. G. (2009). Mode-of-action analysis for induction of rat liver tumours by pyrethrins: Relevance to human cancer risk. *Crit. Rev. Toxicol.* **39**, 501-511.

Qatanani, M. and Moore, D. D. (2005). CAR, the continuously advancing receptor, in drug metabolism and disease. *Curr Drug Metab* **6**, 329-339.

Qatanani, M., Zhang, J. and Moore, D. D. (2005). Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. *Endocrinology* **146**, 995-1002.

Ron, E., Kleinerman, R. A., Boice, J. D., Jr., LiVolsi, V. A., Flannery, J. T. and Fraumeni, J. F., Jr. (1987). A population-based case-control study of thyroid cancer. *J. Natl. Cancer Inst.* **79**, 1-12.

Sugatani, J., Kojima, H., Ueda, A., Kakizaki, S., Yoshinari, K., Gong, Q. H., Owens, I. S., Negishi, M. and Sueyoshi, T. (2001). The phenobarbital response enhancer module in the human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. *Hepatology* **33**, 1232-1238.

Thomas, G. A. and Williams, E. D. (1999). Thyroid stimulating hormone (TSH)-associated follicular hypertrophy and hyperplasia as a mechanism of thyroid carcinogenesis in mice and rats. *IARC Sci. Publ.*, 45-59.

Wei, P., Zhang, J., Egan-Hafley, M., Liang, S. and Moore, D. D. (2000). The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* **407**, 920-923.

Whysner, J., Ross, P. M. and Williams, G. M. (1996). Phenobarbital mechanistic data and risk assessment: Enzyme induction, enhanced cell proliferation, and tumour promotion. *Pharmacol. Ther.* **71**, 153-191.

Yamada, T. (2012). Short-term sudy for mode of action analysis for mouse liver findings by S-

2200TG. Sumitomo Chemical Company Ltd., Study No. S1618, Technical report ROT-0068, Unpublished data.

Yamada, T., Kunimatsu, T., Miyata, K., Yabushita, S., Sukata, T., Kawamura, S., Seki, T., Okuno, Y. and Mikami, N. (2004). Enhanced rat hershberger assay appears reliable for detection of not only (anti-)androgenic chemicals but also thyroid hormone modulators. *Toxicol. Sci.* **79**, 64-74.

Yamada, T., Uwagawa, S., Okuno, Y., Cohen, S. M. and Kaneko, H. (2009). Case study: An evaluation of the human relevance of the synthetic pyrethroid metofluthrin-induced liver tumours in rats based on mode of action. *Toxicol. Sci.* **108**, 59-68.

Yamamoto, Y., Moore, R., Goldsworthy, T. L., Negishi, M. and Maronpot, R. R. (2004). The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumour promotion by phenobarbital in mice. *Cancer Res.* **64**, 7197-7200.

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Document Title

Updated interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study

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CONTENTS

Page

1. Summary

S-2200 Technical Grade (abbreviated as S-2200TG in this document) is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. S-2200TG was not genotoxic in a battery of *in vitro* and *in vivo* assays. The tumourigenic potential of S-2200TG was studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by authorities.

An increased incidence of ovary sex-cord stromal tumour (SCST) was observed in female rats; the increased incidence exceeded the historical control ranges for this strain of rats; therefore, a causal relationship between S-2200TG administration and the ovary tumour induction was not ruled out. In the mouse study, the number of tumours in any tissue did not increase by exposure to S-2200TG. Therefore, one tumour type (benign) in one sex (female) of one species (the rat) occurred in one study. Four and six cases of benign ovarian SCST occurred in female rats exposed to 7000 and 15000 ppm (475 and 1016 mg/kg/day) S-2200TG, respectively. These were dose levels at which body weight gain reduced by > 20%, indicating that the physiology of the rat was sufficiently stressed so that endocrine status may be abnormal. Sex-cord stromal hyperplasia is quite common in aged Wistar rats, and the animals used in the 2-year study with S-2200TG appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were well within historical controls for all groups, and there was no statistical difference between groups for hyperplasia, tumours, or hyperplasia plus tumours. Higher survival rates in the two higher groups may contribute to the higher number of ovarian tumours. The known modes of action via endocrine imbalance are unlikely, evidenced by no interaction with the estrogen receptor and steroidogenesis by *in vitro* assays, no direct ovarian toxicity, and no reproductive abnormality. Furthermore, there was no accumulation or persistence of S-2200 and its metabolites in the ovary. Thus, the sex-cord stromal lesions are unlikely to be direct effects by treatment with S-2200TG.

Based on these considerations, the increased incidence of the SCST observed in the rat 2-year study is not toxicologically significant. Therefore, the overall conclusion is that the data do not suggest a carcinogenic effect of S-2200TG and thus its classification is not warranted.

2. Introduction

S-2200TG is a candidate novel strobilurin fungicide developed by Sumitomo Chemical Co., Ltd. The tumourigenic potential of S-2200TG has been studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice and the test protocols designated by the European Community (EC), Organisation for Economic Co-operation and Development (OECD), US. Environmental Protection Agency (US.EPA), and Ministry of Agriculture, Forestry and Fisheries of Japan (Japan MAFF). These studies have been conducted at Covance Laboratories Ltd, Harrogate, North Yorkshire, England. No tumorigenic findings were observed in mice. An increased incidence of benign ovarian sex-cord stromal tumour (abbreviated as SCST) was observed at the terminal sacrifice in the rat 2-year study.

In this document, the toxicological significance of the slightly higher incidence of benign ovarian SCST in rats is discussed based on existing data of S-2200TG, the usual background of ovarian proliferative change present in elderly rats, and published information. Based on these considerations, the increased incidence of the SCST observed in the rat 2-year study is not

toxicologically significant. Therefore, the overall conclusion is that the data do not suggest a carcinogenic effect and thus classification is not warranted.

3. Genotoxicity

S-2200TG was not genotoxic in a battery of *in vitro* and *in vivo* assays: reverse mutation test in a bacterial system, gene mutation test in Chinese Hamster V79 cells, chromosomal abbreviation test in Chinese Hamster lung cells (CHL/IU), and micronucleus test in CD-1 mice.

4. Metabolism in rats

The absorption, metabolism, distribution and excretion of [benzyl-^{14}C]- and [phenoxy-^{14}C]S-2200 have been investigated in rats. S-2200 is well absorbed (>90%) following oral administration, and is extensively metabolized with effective first pass effect, without any differences in metabolic profiles between [benzyl-^{14}C]- and [phenoxy-^{14}C]S-2200. Subsequent distribution of metabolites of S-2200 is widespread, with relatively high ^{14}C concentration in liver, kidney, pancreas, fat, uterus, ovaries, lung, and adrenals. However, elimination of total radioactivity was rapid, with >95% of the administrated dose being recovered in excreta within 24 hours, and elimination of radioactivity from the plasma and these tissues was almost complete at 168 hours post-administration. Faecal elimination, via the bile, was the primary route of elimination from the body of the rat. In addition, renal elimination was also important for the excretion of metabolites. There was no marked gender-related difference in absorption, distribution, metabolism or excretion.

Therefore, these findings suggest that accumulation and persistence of S-2200 or its metabolites in the ovary inducing ovarian dysfunctions is unlikely to occur in the rat 2-year bioassay.

5. Carcinogenicity study in mouse

Male and female Crl:CD1(ICR) mice were fed 0 (control), 700, 2000, or 7000 ppm S-2200TG (purity, 93.4%) in the diet for 78 weeks (average chemical intakes: 83, 239, and 824 mg/kg/day for males; 99, 280, and 994 mg/kg/day for females, respectively). There were no adverse effects observed. Therefore, the No Observed Adverse Effect Level (NOAEL) for this study was considered to be 7000 ppm (824 mg/kg/day for males and 994 mg/kg/day for females) following 78 weeks of treatment. There were no effects on survival/mortality or on the incidence or morphology of tumours to indicate any oncogenic potential.

6. Carcinogenicity study in rat

6-1. Study design

Male and female Crl:WI(Han) rats were fed 0 (control), 400, 2000, 7000, or 15000 ppm S-2200TG (purity, 93.4%) in the diet for 104 weeks (average chemical intakes: 21, 105, 376, and 804 mg/kg/day for males; 27, 135, 475 and 1016 mg/kg/day for females, respectively). In a previous GLP study "S-2200 Technical Grade: 13 Week Oral (Dietary) Administration Toxicity Study in the Rat (Covance Study Number 0333/290; Dose levels of 0, 800, 4000, 10000 and 20000 ppm; 12 animals/group/sex), there was an overall decrease in body weight gain for males and females given 20000 ppm and an increase in total plasma cholesterol for males and females

given 10000 and 20000 ppm, respectively. Liver weights increased in males given ≥4000 ppm and females given ≥10000 ppm. Microscopically, liver hepatocyte hypertrophy was recorded for animals given 4000, 10000 or 20000 ppm, and the incidence of thyroid follicular cell hypertrophy was higher in animals given 4000, 10000 or 20000 ppm. In addition, the severity of hyaline droplets was higher in the kidney of males given 10000 or 20000 ppm. Due to the decrease in body weight gain, the magnitude of liver weight changes, a higher degree of hepatocyte hypertrophy and increased blood biochemistry parameters (total cholesterol and gamma glutamyltransferase) at 20000 ppm, the overall No-Observed-Adverse-Effect-Level (NOAEL) for the study was considered to be 10000 ppm.

Based on consultation with US.EPA and the Health Canada Pest Management Regulatory Agency (PMRA) regarding MTD (Maximum Tolerated Dose) determination for long-term studies, 15000 ppm was selected to be the highest dose level for the 2-year bioassay. To examine the dose response and no-adverse effect level, 400, 2000 and 7000 ppm were selected for the lower dose levels according to a common ratio of approximately 2 to 5.

6-2. Incidence of ovary tumours

As shown in Figure 1 and Table 1, the incidence of benign ovarian SCST was higher at 7000 and 15000 ppm than control; however, pair wise comparisons were not statistically significant by the Fisher exact test. For tests of increasing dose response by Peto analysis, benign SCST in the ovary of females were statistically significant in treated animals vs. controls (P=0.005). These incidences exceeded the historical background range (0-3.1%, Table 2). Malignant SCST was not observed in any group.

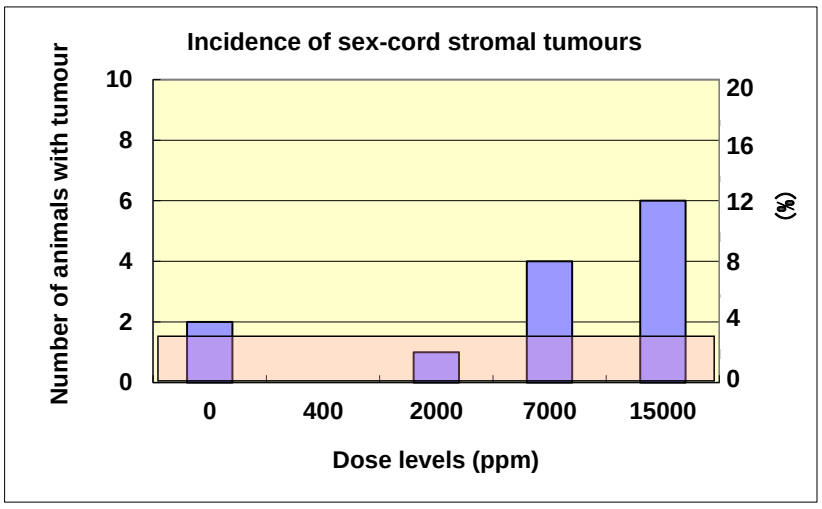

Figure 1. Incidence of sex-cord stromal tumours in the rat 2-year study with S-2200TG.

Highlighted area presents historical background range (0-3.1%).

Fisher exact test; Not significant (p>0.05), Peto test; Significant (p=0.005).

Table 1. Incidence of microscopic findings in the rat 2-year study with S-2200TG

Finding	Level (ppm) / No. examined:	0 / 50	400 / 50	2000 / 50	7000 / 50	15000 / 50
Benign sex-cord stromal tumour (SCST)		2	0	1	4	6
	(%)	4	0	2	8	12
Malignant sex-cord stromal tumour		0	0	0	0	0
	(%)	0	0	0	0	0
Sex-cord stromal hyperplasia		3	8	5	6	5
	(%)	6	16	10	12	10
Combined sex-cord stromal tumour and hyperplasia#		5	8	6	8	9
	(%)	**10**	**16**	**12**	**16**	**18**

Fisher exact test; Not significant (p>0.05), Peto test; Significant (p=0.005).

#: the number of animal exhibiting both hyperplasia and tumour is counted as 1.

Historical control data at Covance UK is shown Table 2.

- Sex-cord stromal hyperplasia: 27.1% [120/442]; range 2 - 48% [1/50 - 31/64].
- Sex-cord stromal tumour - benign: 0.68% [3/442]; range: 0 – 3.1% [0/100 - 2/64] (This tumour was only recorded in two studies out of seven; the incidence was 1/70 and 2/64).

Table 2. Historical background values in females of the rat 2-year studies at Covance UK.

Study ID	1	2	3	4	5	6	7	Total Average
Completed year	2000	2001	2003	2004	2005	2007	2008	2000-08
Pathologist	A	B	B	C	A	D	D	A, B, C, D
Number of animals examined	70	50	50	50	100	60	100	480
Survival rate (%)	78	74	72	72	79	58	45	68
Sex-cord stromal hyperplasia#	31/70	14/49	1/50	1/50	31/64	16/59	26/100	120/442
(%)	44	29	2	2	48	27	26	27.1
Sex-cord stromal tumour#	1/70	0/49	0/50	0/50	2/64	0/59	0/100	3/442
(%)	1.4	0	0	0	3.1	0	0	0.68

#: Data represent the number of animal exhibiting findings compared to the total number of animals examined.

Pathologist of the rat 2-year study with S-2200TG is E but not A~D.

Figure 2 and Table 3 show incidence and severity of sex-cord stromal hyperplasia with gradable differentiation. The number of animals with sex-cord stromal hyperplasia was increased in all treatment groups, but not in a dose-related manner. All of these incidences were within the historical background range (2-48%, Table 2).

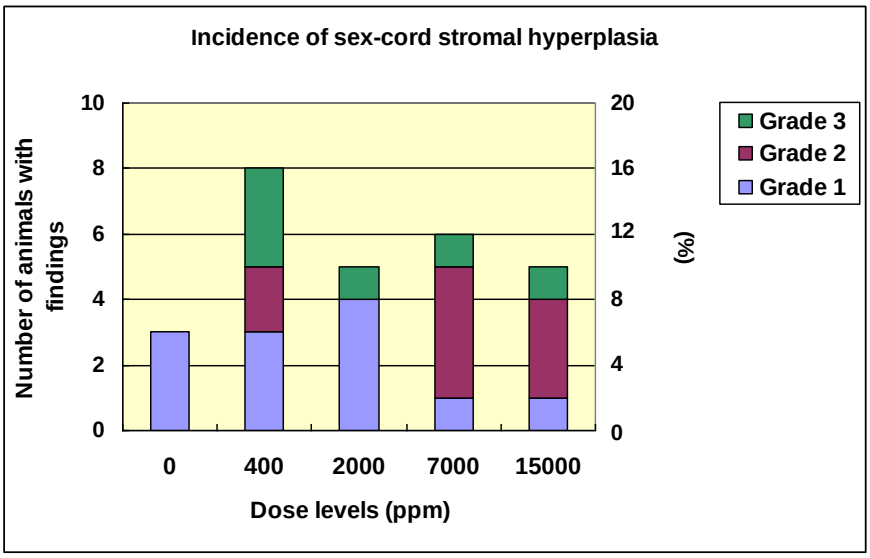

Figure 2. Incidence and severity of sex-cord stromal hyperplasia in the rat 2-year study with S-2200TG.

Table 3. Incidence and severity of sex-cord stromal hyperplasia in the rat 2-year study with S-2200TG

Finding	Level (ppm) / No. examined:	0 50	400 50	2000 50	7000 50	15000 50
Sex-cord stromal hyperplasia		3	8	5	6	5
Grade 1		3	3	4	1	1
Grade 2		0	2	0	4	3
Grade 3		0	3	1	1	1
	(%)	6	16	10	12	10

Grade 1, minimal; Grade 2, slight; Grade 3, moderate.

Mann-Whitney U-test; Not significant ($p>0.05$).

6-3. Assessment of significance

When assessing the overall level of concern of the ovarian SCST observed in the rat S-2200TG carcinogenicity study, some important factors need to be taken into consideration for evaluation of carcinogenic potential.

(a) Tumour type and background incidence;

(b) Multi-site responses;

(c) Progression of lesions to malignancy;

(d) Reduced tumour latency;

(e) Whether responses are in single or both sexes;

(f) Whether responses are in a single species or several species;

(g) Structural similarity to a substance(s) for which there is good evidence of carcinogenicity;

(h) Routes of exposure;

(i) Comparison of absorption, distribution, metabolism and excretion between test animals and humans;

(j) The possibility of a confounding effect of excessive toxicity at test doses;

(k) Mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity.

Regarding S-2200TG, these factors are evaluated below.

(a) Tumour type and background incidence;

Ovarian tumours in rodents can be subdivided into five broad categories including epithelial tumours, sex-cord/stromal-cell–derived tumours, germ cell tumours, tumours derived from nonspecialized soft tissues of the ovary, and tumours metastatic to the ovary from distant sites. Sex-cord/stromal cell-derived tumours include granulosa cell tumour, Sertoli cell tumour, thecoma and SCST (mixed). Ovarian SCST is recognized as a rare tumour amongst human ovary tumours (Scully, *et al.*, 1998) but is the most common spontaneous and induced rat ovarian neoplasm (Tsubota *et al.*, 2004). Because ovarian SCST are morphologically heterogeneous neoplasms that are relatively infrequently encountered, their diagnosis can be difficult. The diagnostic features of ovarian SCST are defined by the International Agency for Research on Cancer (IARC) (Mohr, 1997) as follows:

- Tumour consists of a mixture of granulosa, luteal, theca, Sertoli, and stromal cells, which may show various degrees of differentiation. No cell type dominates (>70%).
- Discrete, well demarcated focal lesions, which are bigger than one large corpus luteum.
- Included in this category are also extremely large, diffuse, mixed-type lesions which encompass the whole ovary and are in size/diameter markedly larger than a normal ovary (old age type sex-cord stromal hyperplasia).

Therefore, focal discrete lesions larger than a large corpus luteum are, in the absence of any other neoplastic morphological criteria, considered to be a tumour. Diffuse mixed-type lesions occasionally become very large. They may encompass the major part of the ovary

and have a size larger than a normal ovary. In these cases they are arbitrarily registered as tumour rather than hyperplasia. Hyperplasia of the sex-cord-stromal cells is quite common in aged Wistar rats and probably other strains of rats but tends not to be diagnosed by some pathologists because it is viewed as a normal aging change. It is seen most commonly in 2-year carcinogenicity studies (Dixon *et al.*, 1999).

In the rat carcinogenicity study with S-2200TG, ovarian SCST were observed in 4/50 (8%) and 6/50 (12%) females of the 7000 and 15000 ppm groups (see Tables 1-3, Figure 1). The incidences (8 and 12%) were higher than that from the historical control range (0-3.1%) derived from several studies of 24-months duration. However, the incidence of the ovarian SCST in the control group of this study (2/50=4%) was higher than the highest level of the historical control range (3.1%; but zero in the most studies, see Table 2), suggesting that animals used in this study were derived from a batch susceptible for ovarian SCST.

The incidences of sex-cord stromal hyperplasia were similar in all groups and within the range of historical controls for this lesion (see Tables 1-3, Figure 2). The historical control data for sex-cord stromal hyperplasia and SCSTs in Table 2, indicates that SCSTs occur only in the presence of the highest rates of sex-cord stromal hyperplasia; which would imply some sort of progressive mechanism from hyperplasia to tumour. In contrast, the absence of a clear dose relationship in incidence or severity of hyperplasia in the current study (Figure 2) provides further support for the tumours to have arisen from a mechanism not involving hyperplasia, and thus are likely to be a chance occurrence.

Since the distinction between hyperplasia and tumour is somewhat arbitrary, based on size, the overall number of sex cord stromal proliferative lesions (hyperplasia plus tumour) provides a more reasonable assessment of a possible effect. For S-2200TG, the combined incidences of SCST and hyperplasia (Figure 3) were within the historical control incidences for hyperplasia alone (average, 120/442=27.1%; range, 2 - 48%; see Table 2), there is no increase in incidence of the combined sex-cord stromal proliferative lesions, and the incidences in all groups are well within the historical control range (actually less than the mean). The lack of statistical significance for hyperplasia, tumours, or hyperplasia plus tumours further supports the conclusion that these are not treatment related.

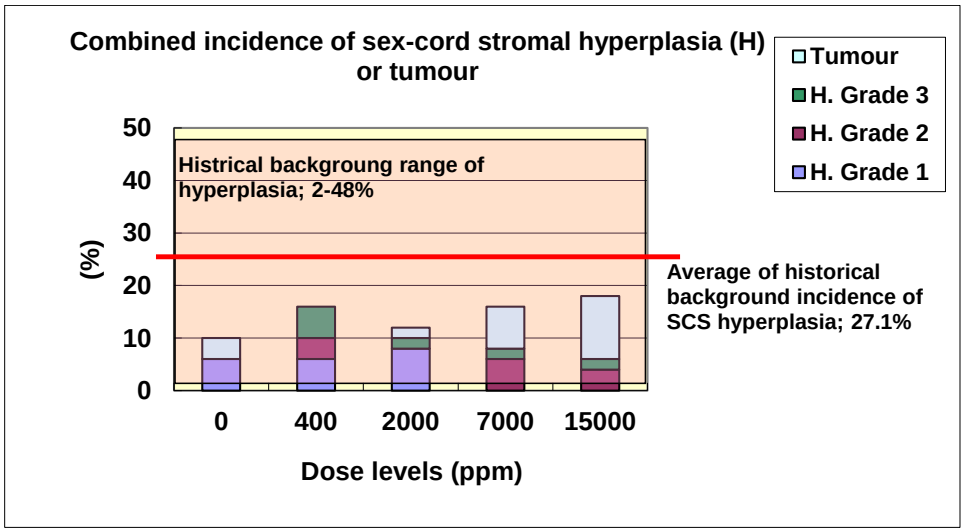

Figure 3. Combined incidence of sex-cord stromal hyperplasia and tumour in the rat 2-year study with S-2200TG.

The animal exhibiting both hyperplasia and tumour is counted as animals exhibiting tumour. Mann-Whitney U-test; Not significant ($p>0.05$).

(b) Multi-site responses;

The increase of tumour incidence was only observed in a single-site (ovary). There was no evidence of tumour multiplicity observable in the ovary in the rat 2-year study with S-2200TG.

(c) Progression of lesions to malignancy;

The ovarian SCSTs observed in the rat 2-year study with S-2200TG were all benign tumours. No malignant SCST was observed. Therefore, there was no evidence of progression to malignant tumour.

(d) Reduced tumour latency;

Since most SCSTs were observed in animals sacrificed after two years of treatment with S-2200TG but not observed at an interim sacrifice after one year of treatment, the tumours appeared at a late stage of treatment, after 1.5 years of treatment based on unscheduled death/sacrifice animals: in the 15000 ppm group, no SCSTs were observed in any of the 9 unscheduled death/sacrificed animals during Weeks 57 to 98. At 7000 ppm, one unscheduled sacrificed animal at Week 98 had ovarian SCS hyperplasia and tumour; no SCSTs were observed in the other 9 unscheduled death/sacrificed animals during Weeks 57 to 102. Therefore, reduced tumour latency was not observed in the rat 2-year study with S-2200TG.

(e) Whether responses are in single or both sexes;

A higher incidence of tumour with possible treatment-related effect was observed in the ovary but not in the testis; three animals bearing testicular Leydig cell tumour were observed

at 15000 ppm (3/50=6%), but this was within the historical background range in the laboratory (0-6%) and there was no increased incidence of interstitial cell hyperplasia of the testis. Therefore, the response was observed only in a single sex (female).

(f) Whether responses are in a single species or several species;

A higher incidence of the ovarian SCST was observed in rat but not in mouse. There were no increased incidences of any tumour type in the mouse. Therefore, the response was observed in a single species (rat). Thus, this is a slight increase in the incidence of a benign tumour at a single site in a single sex in a single species.

(g) Structural similarity to a substance(s) for which there is good evidence of carcinogenicity;

S-2200TG is a candidate novel strobilurin fungicide. Of known strobilurin chemicals, none increase ovarian tumours in rats or mice.

(h) Routes of exposure;

In the carcinogenicity study, rats were treated with S-2200TG fed in the diet. The oral route was selected because it is one of the potential exposure routes for humans.

(i) Comparison of absorption, distribution, metabolism and excretion between test animals and humans;

Three metabolites (5-CH_2OH-S-2200, 2-CH_2OH-S-2200, and 4-OH-S-2200) were formed in liver S9 fraction of humans, rats and mice. No species related difference was observed in the three metabolites. Data for absorption, distribution and excretion of S-2200 are not available in humans.

(j) The possibility of a confounding effect of excessive toxicity at test doses;

Four and six cases of benign ovarian SCST were observed in female rats exposed to 7000 and 15000 ppm of S-2200TG, respectively. As shown in Figure 4 and Table 4, body weight gain was suppressed by more than 10% after Week 40 at 7000 ppm, and throughout the treatment period at 15000 ppm. Maximal suppression reached more than 20%, indicating that these doses considerably exceeded the maximum tolerated dose. 2000 ppm of S-2200TG also showed weight suppression of more than 10%, but it was only observed at a later period of treatment (i.e., after Week 68).

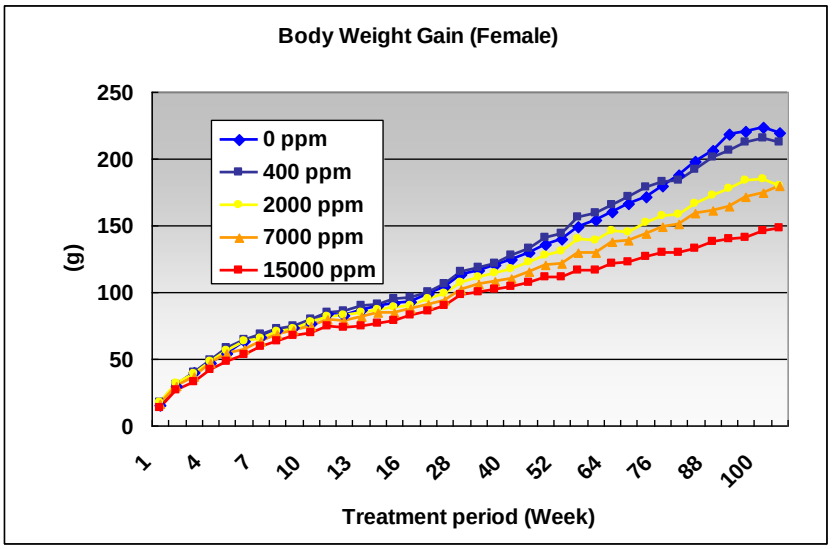

Figure 4. Alteration of body weight gain in female rats treated with S-2200TG. Data present group mean value.

Table 4. Relative suppression of body weight gain in female rats treated with S-2200TG.

Week	Dose levels of S-2200TG			
	400 ppm	2000 ppm	7000 ppm	15000 ppm
1	12	18	6	-12
2	2	5	-3	-13
3	1	-2	-7	-17
4	6	2	1	-10
5	8	4	0	-11
6	2	0	-9	-16
7	4	0	-4	-10
8	1	-1	-3	-12
9	2	-1	-3	-8
10	4	1	-2	-10
11	2	-3	-5	-11
12	3	-1	-5	-12
13	3	-3	-7	-14
14	1	-3	-6	-15
15	3	-4	-7	-14
16	3	-3	-5	-11
20	1	-4	-8	-13
24	2	-5	-10	-15
28	2	-5	-10	-13
32	2	-4	-8	-14
36	1	-4	-10	-15
40	2	-6	-11	-17
44	3	-6	-11	-17
48	3	-6	-11	-18
52	3	-7	-14	-21
56	5	-7	-13	-22
60	3	-10	-15	-24
64	3	-9	-14	-24
68	3	-13	-17	-26
72	4	-11	-16	-26
76	1	-13	-17	-28
80	-2	-16	-20	-31
84	-3	-16	-20	-33
88	-2	-16	-22	-33
92	-5	-19	-25	-36
96	-4	-17	-22	-36
100	-4	-18	-22	-35
104	-3	-18	-18	-33

Values represent percent change from control value.

Highlighted values indicate more than 10% suppression of body weight gain.

The highest dose in the bioassay needs to induce minimal toxicity, such as characterized by an approximately 10% reduction in body weight gain (maximal tolerated dose, MTD) (ECHA, 2012; Rhomberg *et al.*, 2007). The MTD is the highest dose of the test agent during the bioassay that can be predicted not to alter the animal's normal longevity from effects other than carcinogenicity. Excessive toxicity, for instance toxicity at doses exceeding the MTD, can affect the carcinogenic responses in bioassays. Tumours occurring only at excessive doses associated with severe toxicity generally have a more doubtful potential for carcinogenicity in humans (ECHA, 2012; Rhomberg *et al.*, 2007).

Survival rate also appears to affect interpretation of results in the bioassay. The common practice of scheduling a terminal sacrifice after 2 years on test reflects a judgment that this is optimal for detecting potential carcinogenic effects (Rhomberg *et al.*, 2007). However, shorter survival risks missing induced tumours and longer survival tends to induce an increasing number of background tumours. Figure 5 presents the survival rate in female rats of the 2-year study with S-2200TG. Survival rate was relatively higher at 7000 and 15000 ppm than control; especially, in the last 6 months of treatment period (Figure 5B).

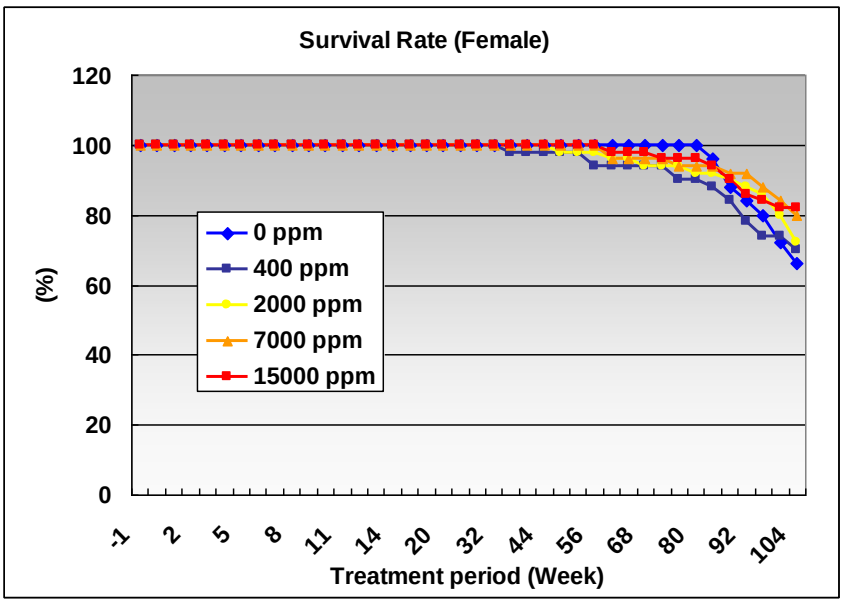

Figure 5A. Survival rate in females of the rat 2-year study with S-2200TG throughout 2-year treatment

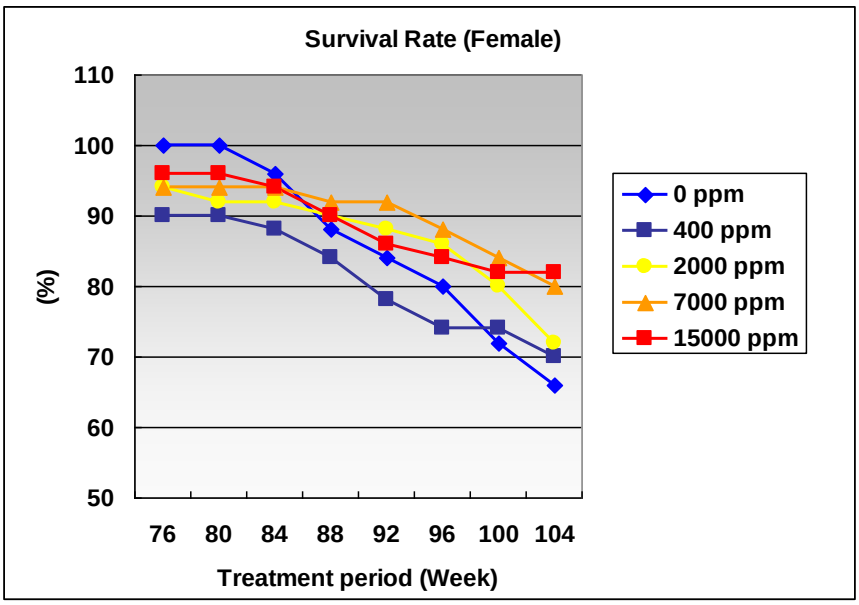

Figure 5B. Survival rate in females of the rat 2-year study with S-2200TG during last 6-month treatment

As shown in Table 5, the number of surviving rats of the 7000 ppm and 15000 ppm female groups at terminal sacrifice (40 and 41 rats, respectively) was higher than those of the control female group (33 rats). These values were not statistically significant; p-values of 7000 and 15000 ppm groups by the Fisher exact test were 0.088 and 0.055, respectively. In comparison with historical background data at Covance UK (see Table 2), the survival rate of 7000 and 15000 ppm groups (80 and 82%, respectively) were slightly higher than the control range (45-79%). The higher number of surviving animals may affect the incidence of the SCST as a confounding factor because animals used in this study were derived from a susceptible batch for ovarian SCST as discussed in Section (a).

Table 5. Survival rate in the rat carcinogenicity study with S-2200TG

	Male					Female				
Dose levels (ppm)	**0**	**400**	**2000**	**7000**	**15000**	**0**	**400**	**2000**	**7000**	**15000**
Survivals	**32**	**42***	**32**	**41***	**36**	**33**	**35**	**36**	**40#**	**41##**
Survival rate (%)	**64**	**84**	**64**	**82**	**72**	**66**	**70**	**72**	**80**	**82**

*; $p<0.05$, #; $p=0.088$, ##;$p=0.055$ by Fisher exact test.

(k) Mode of action (MOA) and its relevance for humans

(k-1). Negative findings of genotoxicity studies suggest a non-genotoxic MOA.

(k-2). Ovarian function and dysfunction are intimately linked with the hypothalamus, the pituitary, the uterus, and other endocrine organs. It is known that there are two kinds of

MOA for induction of ovarian tumour including SCST in rodents; they involve direct and secondary effects on the ovary. In both cases, increased gonadotropin is the causative factor for ovary tumour development. The transplantation of ovarian tissue under the splenic capsule in ovariectomized rats caused ovarian tumours, including SCST (Biskind and Biskind, 1949; Biskind *et al.*, 1950; Capen, 2008; Dittrich *et al.*, 2001; Jager *et al.*, 1995). Transplantation in ovariectomized rats caused estrogens produced by the transplanted ovary to drain to the liver. As sex hormone globulin levels are low in adult rats (Dittrich *et al.*, 2001), estradiol is metabolized directly and completely during first passage through the liver so that no estradiol reaches the pituitary gland to down-regulate gonadotropin secretion. The presence of a single functioning gonad prevented the development of the proliferative lesions in the ovary. Furthermore, administration of exogenous estrogen or testosterone after transplantation completely suppressed development of the proliferative changes in the ovarian cortex. Taken together, as shown in Figure 6, lack of negative feedback from estrogen and a high level of gonadotropin stimulation are necessary for the development of these tumours in rats (Biskind and Biskind, 1949; Capen, 2008; Jager *et al.*, 1995).

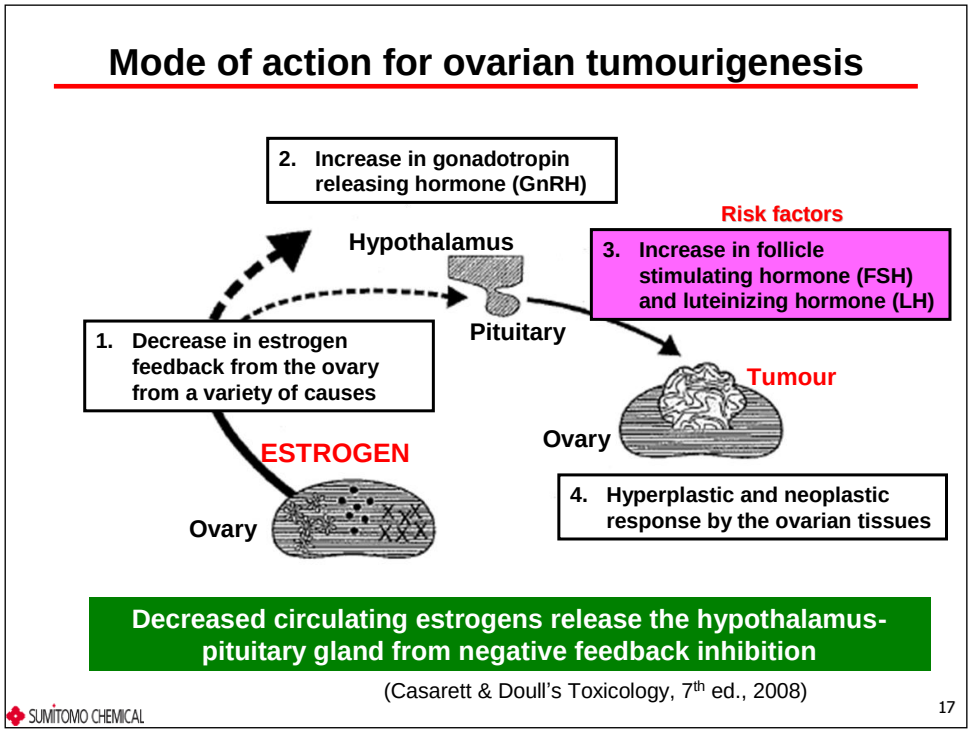

Figure 6. Mode of action for ovarian tumourigenesis in rodents. Figure modified from Casarett & Doull's Toxicology: The basic Science of Poisons (Capen, 2008).

- No interaction with estrogen receptor and no effects on steroidogenesis

  Selective estrogen receptor modulators (SERMs) (e.g., tamoxifen, toremifene, and raloxifene) have been reported to increase the incidence of ovarian tumours when administered chronically to rodents (Capen, 2008; Long *et al.*, 2001). In fact, raloxifene binding to the estrogen receptor induced an elevation of serum Luteinizing hormone (LH) and ovarian tumour in rats (Long *et al.*, 2001).

S-2200TG was negative in an estrogen receptor reporter gene assay (Suzuki, 2012) and assay for steroidogenesis (Kubo, 2012). Unfortunately, hormone levels in peripheral blood could not be determined in the 2-year study. Taken together with no abnormalities in reproductive function in rats or in endocrine organs in general toxicity studies in rats, mice and dogs, it is suggested that secondary effects via endocrine disruption by S-2200TG are unlikely. Especially, in the case of LH, though, the absence of an increase in Leydig cell tumours in the current study is important in supporting the position that LH was not elevated, and thus the SCSTs did not arise by a hormonal mechanism, rather being most likely due to chance. This is particularly likely since the incidences of hyperplasia plus benign tumours (total proliferative lesions of the ovary) were within historical controls.

- No indication of endocrine disruption in the toxicity-studies

Apart from the secondary effect via endocrine disruption, direct effect (cytotoxic effect) on the ovary also involves the lack of negative feedback from estrogen and a consequent high level of gonadotropin. Maronpot (1987) demonstrated that the ovarian lesions documented in the National Toxicology Program (NTP) studies covered in his paper provide supportive evidence that alterations noted in ovaries of treated rodents at the conclusion of a 90-day study may herald the ultimate development of ovarian neoplasia upon continued treatment (Maronpot, 1987). S-2200TG did not provide any evidence indicating ovary lesions or toxicity in any studies in rat, mouse and dog, except for the hyperplasia and tumour at the terminal sacrifice in the rat 2-year study. Furthermore, no abnormality was observed in the rat reproduction study. Therefore, a direct cytotoxic effect of S-2200TG on the ovary is also unlikely.

- Severe suppression of body weight may relate to induction of ovary tumours

A number of studies showed that life-long restriction in caloric intake improves 2-year survival, controls adult body weight and delays the onset of diet- and age-related spontaneous diseases and tumours (Molon-Noblot *et al.*, 2003). Pituitary function is also reduced during fasting or food restriction, and life-long food restriction induced a decline of the incidence of pituitary tumours in rat (Molon-Noblot *et al.*, 2003). Unfortunately, the incidence of ovary tumours was not determined in the study by Molon-Noblot *et al.* (2003). However, Rehm *et al.* (1984) demonstrated that all animals subjected to food restriction live longer and developed more ovarian neoplasms than those fed ad libitum in a mouse study. This finding suggests that a decrease of tumour appearance by life-long dietary restriction did not prove to be true for the present type of ovarian neoplasms, even though the secretion of gonadotropins is impaired under limited feeding conditions (Rehm *et al.*, 1984). Interestingly, McShane and Wise (1996) have shown that moderate life-long caloric restriction, resulting in average body weights that are approximately 76% those of controls, delays reproductive senescence in rats without causing a cessation of regular estrous cycles earlier in life. Furthermore, they demonstrated that LH secretion is enhanced by caloric restriction. Unfortunately, serum LH levels were not able to be determined in the rat 2-year study of S-2200TG, but significant reduction in the incidence of pituitary tumours was observed in females treated with 15000 ppm of S-2200TG together with more than 20% suppression of body weight.

Therefore, the existing data do not support that the higher incidence of the SCST observed in rats treated with S-2200TG is via this well-known MOA involving excess gonadotropin. Thus, the slightly higher incidence of the SCST is not toxicologically significant at most, especially when viewed in the perspective of a high background of proliferative lesions (stromal hyperplasia), a higher survival at dose levels where severe body weight suppression (which may induce abnormal endocrine status) was observed, and that this represents a slight increase of a benign lesion at a single site in a single sex in a single species. Taken together, the evidence supports the conclusion that the ovarian lesions in female rats were not toxicologically significant.

(k-3). No correlation between the ovarian cancer in women and increased serum gonadotropin.

A positive result in a rodent carcinogenicity study should not automatically preclude further development of a compound; future progress in this field should increase the accuracy of the rodent carcinogenicity study as a tool in human safety assessment (Alison *et al.*, 1994). In humans, the concentration of sex hormone-binding globulins is high enough to prevent the fast metabolism of estradiol in the liver (Mueller *et al.*, 2005). Therefore, this suggests that it is difficult to elevate gonadotropin levels in human peripheral blood. Furthermore, although hormonal imbalances in rodents have been shown to alter ovarian morphology and function and are correlated with an increased incidence of ovarian neoplasia, these preclinical models are not predictive of ovarian cancer in women. To date, there is no correlation between the occurrence of ovarian cancer in women and increased serum gonadotropin levels. In fact, women with a personal or family history of ovarian cancer have significantly lower serum LH concentrations than women without such a history. Thus, the relationship between elevated circulating gonadotropin levels and an increased incidence of ovarian tumours in rodents does not appear to be true in humans (Capen, 2008; Long *et al.*, 2001). Stromal tumours are rare in the human ovary (Scully *et al.*, 1998). Furthermore, epidemiology demonstrated that a positive association between ovarian cancer and obesity is well known (Olsen *et al.*, 2007), but not associated with lower body weight. Rather, ovarian cancer is associated with height and, among never-users of hormone therapy, with body mass index (Collaborative Group on Epidemiological Studies of Ovarian Cancer, 2012), and moderate energy restriction during adolescence was associated with a decrease in ovarian cancer risk (Schouten *et al.*, 2011).

Therefore, even if the higher incidence of the SCST in rats treated with S-2200TG is treatment-related, the toxicological significance of this alteration for human safety is questionable.

7. Determination of criteria for classification and labeling for carcinogenicity

Our proposed classification and labeling of S-2200TG for carcinogenicity is as follows:

One tumour type (benign) in one sex (female) of one species (the rat) occurred in one study with S-2200TG. S-2200TG is non-genotoxic, which lowers the level of concern for classification. Four and six cases of benign ovarian sex-cord stromal tumours occurred in female rats exposed to 7000 and 15000 ppm (475 and 1016 mg/kg/day) S-2200TG, respectively. These were dose levels at which body weight gain was reduced by > 20%, indicating that the physiology of the rat was

sufficiently stressed so that endocrine status may be abnormal. The sex-cord stromal hyperplasia is quite common in aged Wistar rats (Dixon *et al.*, 1999), and the animals used in the 2-year study with S-2200TG appear to be derived from a susceptible batch. The incidences of sex-cord stromal proliferative lesions were well within historical controls for all groups, and there was no statistical difference between groups for hyperplasia, tumours, or hyperplasia plus tumours. Higher survival rate in the two higher groups may contribute to the higher number of ovarian tumours. The known modes of action via endocrine imbalance are unlikely, evidenced by no interaction of estrogen receptor and steroidogenesis by *in vitro* assays, no direct ovarian toxicity, and no reproductive abnormality. There was no accumulation or persistence of S-2200 and its metabolites in the ovary. Thus, the sex-cord stromal lesions in female rat are not toxicologically significant.

Given the uncertainties over the significance of the finding, the overall conclusion is that the data do not suggest a carcinogenic effect and thus classification is not warranted.

8. References

Alison, R. H., Capen, C. C. and Prentice, D. E. (1994). Neoplastic lesions of questionable significance to humans. *Toxicol. Pathol.* **22**, 179-186.

Biskind, G. R. and Biskind, M. S. (1949). Experimental ovarian tumors in rats. *Am. J. Clin. Pathol.* **19**, 501-521.

Biskind, G. R., Kordan, B. and Biskind, M. S. (1950). Ovary transplanted to spleen in rats: The effect of unilateral castration, pregnancy, and subsequent castration. *Cancer Res.* **10**, 309-318.

Capen, C. C. (2008). Unit 4. Target organ toxicity. 21. Toxic responses of the endocrine system. 7th ed. (C. D. Klaassen, Ed.). McGraw-Hill Companies, Inc., New York.

Collaborative Group on Epidemiological Studies of Ovarian Cancer. (2012). Ovarian cancer and body size: Individual participant meta-analysis including 25,157 women with ovarian cancer from 47 epidemiological studies. *PLoS Med* **9**, e1001200.

Dittrich, R., Recabarren, S., Mitze, M. and Jaeger, W. (2001). Role of gonadotropins in malignant progression of sex cord stromal tumors produced by sequential auto- and isogenic transplantation of ovarian tissue in ovariectomized rats. *J. Cancer Res. Clin. Oncol.* **127**, 495-501.

Dixon, D., Leininger, J., Valerio, M., Johnson, A., Stabinski, L. and Frith, C. (1999). Proliferative lesions of the ovary, uterus, vagina, cervix and oviduct in rats. STP/ARP/AFIP, Washington, DC.

ECHA (2012). Guidance on the application of the CLP criteria, guidance to regulation (EC) no 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 2.0.

Jager, W., Dittrich, R., Recabarren, S., Wildt, L. and Lang, N. (1995). Induction of ovarian tumors by endogenous gonadotropins in rats bearing intrasplenic ovarian grafts. *Tumour Biol.* **16**, 268-280.

Kubo, H. (2012). In vitro steroidogenesis assay of S-2200TG in H295R cells. Study no. HK001, Sumitomo Chemical Company Ltd., technical report, unpublished data.

Long, G. G., Cohen, I. R., Gries, C. L., Young, J. K., Francis, P. C. and Capen, C. C. (2001). Proliferative lesions of ovarian granulosa cells and reversible hormonal changes induced in rats by a selective estrogen receptor modulator. *Toxicol. Pathol.* **29**, 719-726.

Maronpot, R. R. (1987). Ovarian toxicity and carcinogenicity in eight recent national toxicology program studies. *Environ. Health Perspect.* **73**, 125-130.

McShane, T. M. and Wise, P. M. (1996). Life-long moderate caloric restriction prolongs reproductive life span in rats without interrupting estrous cyclicity: Effects on the gonadotropin-releasing hormone/luteinizing hormone axis. *Biol. Reprod.* **54**, 70-75.

Mohr, U. (1997). International Classification of Rodent Tumours. Part I-The Rat. 9.Female Genital System., The International Agency for Research on Cancer., Lyon.

Molon-Noblot, S., Laroque, P., Coleman, J. B., Hoe, C.-M. and Keenan, K. P. (2003). The effects of ad libitum overfeeding and moderate and marked dietary restriction on age-related spontaneous pituitary gland pathology in sprague-dawley rats. *Toxicol. Pathol.* **31**, 310-320.

Mueller, A., Maltaris, T., Dimmler, A., Hoffmann, I., Beckmann, M. W. and Dittrich, R. (2005). Development of sex cord stromal tumors after heterotopic transplantation of cryopreserved ovarian tissue in rats. *Anticancer Res.* **25**, 4107-4111.

Olsen, C. M., Green, A. C., Whiteman, D. C., Sadeghi, S., Kolahdooz, F. and Webb, P. M. (2007). Obesity and the risk of epithelial ovarian cancer: A systematic review and meta-analysis. *Eur. J. Cancer* **43**, 690-709.

Rehm, S., Dierksen, D. and Deerberg, F. (1984). Spontaneous ovarian tumors in han:Nmri mice: Histologic classification, incidence, and influence of food restriction. *J. Natl. Cancer Inst.* **72**, 1383-1395.

Rhomberg, L. R., Baetcke, K., Blancato, J., Bus, J., Cohen, S., Conolly, R., Dixit, R., Doe, J., Ekelman, K., Fenner-Crisp, P., Harvey, P., Hattis, D., Jacobs, A., Jacobson-Kram, D., Lewandowski, T., Liteplo, R., Pelkonen, O., Rice, J., Somers, D., Turturro, A., West, W. and Olin, S. (2007). Issues in the design and interpretation of chronic toxicity and carcinogenicity studies in rodents: Approaches to dose selection. *Crit. Rev. Toxicol.* **37**, 729-837.

Schouten, L. J., van Dijk, B. A. C., Lumey, L. H., Goldbohm, R. A. and van den Brandt, P. A. (2011). Energy restriction during childhood and early adulthood and ovarian cancer risk. *PLoS ONE* **6**, e27960.

Scully, R. E., Young, R. H. and Clement, P. B. (1998). Tumors of the Ovary, Maldeveloped Gonads, Fallopian Tube, and Broad Ligament. Atlas of Tumor Pathology, Fascicle 23, Third Series, Armed Forces Institute of Pathology, Washington, DC, p.169-226.

Suzuki, N. (2012). Evaluation of effects of S-2200TG and its metabolites on human estrogen receptor alpha and human androgen receptor using *in vitro* reporter gene assays. Study no. RGA-130, Sumitomo Chemical Company Ltd., technical report, unpublished data.

Tsubota, K., Yoshizawa, K., Fujihira, S., Okazaki, Y., Matsumoto, M., Nakatsuji, S. and Oishi, Y. (2004). A spontaneous ovarian immature teratoma in a juvenile rat. *J. Toxicol. Pathol.* **17**, 211-218.

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